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A STUDY OF THE ROLE OF THIAMINE AND BIOTIN IN THE METABOLISM OF RUMEN MICROORGANISMS

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ABSTRACT

Experiments were conducted to determine the role of some of the B vitamins in the production of volatile fatty acids by rumen organisms *in vitro*.

The antimetabolites oxythiamine HCl, neopyrithiamine HBr, or desthiobiotin at concentrations of up to 4 mM, 2 mM, or 0.48 mM respectively in the fermentation mixture did not influence cellulose digestion or volatile fatty acid production. Thiopental sodium (0.72 mM) inhibited acetate and propionate production equally while 0.18 mM hexetidine, in one run, caused a greater decrease in acetate than in propionate production; this would be expected if this compound inhibited the function of thiamine in pyruvate decarboxylation.

The addition of 12 units of avidin to the fermentation medium, or the deletion of biotin from the medium resulted in a marked decrease in cellulose digestion and volatile fatty acid production. The production of propionate was decreased to a greater extent than the production of acetate.

Deletion of p-aminobenzoic acid from the *in vitro* medium resulted in a greater decrease in propionate than in acetate production. Usually more propionate than acetate was produced per unit of cellulose digested. These observations suggest that a primary effect of biotin deficiency was the inhibition of cellulose digestion.

Glucose, 3-phosphoglycerate, pyruvic acid, lactic acid, oxaloacetic acid, malic acid, fumaric acid and succinic acid were converted to volatile fatty acids by the rumen organisms. Glucose and lactic acid were converted to acetate, propionate and butyrate. Lactic acid gave rise to a large proportion of butyrate. Pyruvic acid and 3-phosphoglycerate were converted almost exclusively to acetate. Oxaloacetic acid was converted to

acetate only. Malic and fumaric acids were largely converted to propionate although there was some conversion to acetate. Succinic acid was converted almost entirely to propionate.

Oxaloacetic acid and 3-phosphoglycerate reduced cellulose digestion. The reduction due to 3-phosphoglycerate was not as large as that produced by oxaloacetic acid.

Neither the effects of biotin deficiency on the conversion of the intermediates to volatile fatty acids nor the effects of the intermediates on cellulose digestion during biotin deficiency provided evidence for a biotin-block in any of the potential pathways from glycolytic intermediates to fatty acids.

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INTRODUCTION

The major end products of the rumen fermentation of dietary carbohydrate are the steam-volatile fatty acids. These short-chain, organic acids are of major importance in the energy economy of the ruminant animal. Factors affecting the proportions in which the volatile fatty acids are formed in the rumen are of major concern to ruminant nutritionists, because it has been established that efficiency of energy utilization varies according to the composition of the fatty acid mixture formed in the rumen. The individual fatty acids have characteristic effects upon some production traits, such as per cent fat in milk.

A considerable amount of research has been devoted to the study of the routes of acetic and propionic acid formation in pure species of bacteria but there has been very little experimental work done on the routes of formation of these acids in the rumen.

The B vitamins are synthesized in the rumen. Results of pure culture work indicate that some of the B vitamins are involved in the metabolic pathways leading to volatile fatty acids.

Experimental study of the biochemical conversions involved in the production of volatile fatty acids by the mixed bacterial population of the rumen and of the relationship of thiamine and biotin to these conversions would be of value. It was reasoned that the role of the vitamins in the pathways of volatile fatty acid production and in cellulose digestion could best be studied if antagonists of the vitamins were added to media used to incubate rumen microorganisms *in vitro*.

REVIEW OF LITERATURE

A. Pathways of Volatile Fatty Acid Formation in the Rumen

I. The Proposed Central Role of Glycolysis

The carbohydrates presented as substrates to the rumen population vary greatly in the extent to which they are polymerized. The monosaccharide units are of various carbon chain lengths and at various levels of oxidation (Annison and Lewis, 1959; Halliwell, 1961; Barnett and Reid, 1961). Most of the carbohydrates that have been identified in ruminant diets have been shown to be fermented by the rumen population (Howard, 1959). The major end products of the fermentation of carbohydrates by the mixed rumen population are the volatile fatty acids (VFA). It would be expected that the carbon of the substrates would be channeled through a common biochemical pathway prior to incorporation into the end products.

The reactions of the glycolytic pathway of carbohydrate degradation are known to play a very central role in the metabolism of numerous organisms. The glycolytic pathway would be expected to be of central importance in the metabolism in the rumen, in that it would be via these reactions that carbon would be directed towards the formation of VFA.

Kitts and Underkofler (1954), using the inhibitors toluene, thymol or sodium flouride to prevent the further metabolism of the initial cleavage products, demonstrated the production of glucose as a step in the degradation of cellulose by rumen organisms.

Nasr (1950) has demonstrated the presence of α-amylase in rumen liquor, suggesting that starch would be degraded eventually to maltose. Maltose was metabolized by rumen bacteria (Meites, Burrell and Sutton, 1951) presumably via conversion to glucose.

The most abundant hemicelluloses are xylans (Halliwell, 1961),

the fermentation of which results in the production of xylose as an intermediate step (Pazur et al., 1957).

Most of the oligosaccharides that would reach the rumen would be converted to intermediates of glycolysis during their metabolism.

The major monosaccharides resulting from the metabolism of the carbohydrate fraction of the ruminant ration would be glucose and xylose.

II. Possible Routes From Intermediates of Glycolysis to VFA on the Basis of Pure Culture and Enzyme Studies

a. Routes of Acetate Production

1. Decarboxylation of Pyruvate

In established biochemical systems, the glycolytic precursor of acetate or acetate derivatives, is pyruvate. The overall conversion of pyruvate to acetate will be referred to hereafter as Reaction 1. It has been shown that in yeast, pyruvate can be nonoxidatively decarboxylated, giving rise to acetaldehyde or an active intermediate thereof. In acetic acid bacteria, the acetaldehyde is oxidized to acetic acid by means of nicotinamide adenine dinucleotide-, or nicotinamide adenine dinucleotide phosphate-linked acetaldehyde dehydrogenase (DeLey and Schell, 1962). In animal tissues pyruvate is known to be oxidatively decarboxylated to acetyl-Coenzyme A (acetyl-CoA). Pyruvate decarboxylation requires thiamine pyrophosphate (TPP) as a cofactor in yeast and animal tissues (Cantarow and Schepartz, 1962).

Studies have recently been carried out on the degradation of pyruvate by Micrococcus lactilyticus[1]. McCormick, Ordal and Whiteley (1962a) prepared cell-free extracts of M. lactilyticus and found that at pH 8.5, the extracts degraded pyruvate stoichiometrically to formate and acetyl phosphate. At the alkaline pH, the bacterial extracts also catalyzed a rapid exchange between the carboxyl group of pyruvate and formate. At pH 6.5, the M. lactilyticus extracts degraded pyruvate to acetyl phosphate, CO_2 and H_2 and

[1]very similar to, if not identical with, the rumen organism Veillonella gazogenes.

a rapid exchange between the carboxyl group of pyruvate and CO_2 was catalyzed. TPP was required by M. lactilyticus extracts for pyruvate degradation. It could not be demonstrated that the extracts required CoA to accomplish the degradation of pyruvate at basic pH (McCormick et al., 1962b). These workers also found that the exchange of CO_2 with pyruvate carboxyl catalyzed by these extracts was not influenced by avidin or biotin. M. lactilyticus extracts required CoA in pyruvate degradation at acid pH.

Cell-free extracts of the rumen microorganism isolated by Elsden et al. (1956) and identified only as LC, were prepared by Peel (1960). Pyruvate was converted to CO_2, H_2 and acetate by these extracts under anaerobic conditions. Partially purified preparations of the extract were found capable of reducing 2,6-dichlorophenolindophenol and ferricyanide very rapidly, while crude extracts would reduce flavin mononucleotide, methylene blue and 2,3,5-triphenyltetrazolium less rapidly, upon addition of pyruvate. Pyridine nucleotides were not reduced by the crude extract of LC with pyruvate as the substrate. The reduction of the dyes by the LC preparations simply eliminated the final hydrogenase reaction which would normally yield H_2. CoA was required for pyruvate degradation by LC extracts, but acetyl phosphate rather than acetyl-CoA was found in the final mixture. It was suggested that the immediate product of the pyruvate degradation was acetyl-CoA, which was converted to acetyl phosphate, by means of a phosphotransacetylase. After extensive dialysis of LC preparations, TPP did not stimulate pyruvate degradation. This was not a strict demonstration that TPP was not required for pyruvate breakdown, but it indicated that if TPP was a cofactor it was quite firmly bound to the apoenzyme.

Shuster and Lynen (1960) carried out a study of the exchange between pyruvate and CO_2 catalyzed by ammonium sulfate fractions of cell-free extracts of Clostridium kluyveri. Extended dialysis destroyed the

exchange activity of the preparations but restoration of 70 to 80 per cent of the activity was achieved upon addition of TPP, adenosine triphosphate (ATP) and Mg^{++}. The exchange catalyzed by Cl. kluyveri extracts was not stimulated by the addition of orthophosphate or CoA. The exchange reaction was very drastically inhibited by avidin; an effect which was reversed by prior incubation of the avidin with biotin. Biotin alone did not stimulate CO_2-pyruvate exchange. Thus, on the basis of these results, and using intermediates that have been reported in the literature (Holzer et al., 1962) the following series of reactions was proposed by Shuster and Lynen (1962) to partially account for pyruvate degradation by Cl. kluyveri.

pyruvate + TPP $\rightleftharpoons$ α-pyrophosphoryl-thiaminyl-lactic acid

α-pyrophosphoryl-thiaminly-lactic acid + biotin-enzyme $\rightleftharpoons$ α-hydroxyethyl-TPP + CO_2-biotin-enzyme

CO_2-biotin-enzyme + ADP[1] + Pi[2] $\rightleftharpoons$ ATP + biotin-enzyme + CO_2

Sum: Pyruvate + TPP + ADP $\rightleftharpoons$ " α-hydroxyethyl-TPP" + CO_2 + ATP

The role of biotin as a CO_2 acceptor is identical to its role in the carboxylation reactions catalyzed by acetyl carboxylase and propionyl carboxylase. Biotin had not previously been directly linked with pyruvate decarboxylation.

Stern (1963) reported results of studies concerned with pyruvate degradation using extracts of Clostridium kluyveri. Using $C^{14}O_2$ he found that extracts of Cl. kluyveri fixed CO_2 though condensation with pyruvate giving rise to oxaloacetate and through exchange with the carboxyl group of pyruvate. The oxaloacetate-producing reaction was inhibited by avidin, independent of cocarboxylase and required ATP and Mg^{++} or Mn^{++}. This reaction is very similar to that catalyzed by pyruvate carboxylase of Aspergillus niger (Bloom and Johnson, 1962). The cofactor requirements of the two reactions are also similar, including a requirement for biotin.

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1 adenosine diphosphate
2 inorganic phosphate

Pyruvic carboxylase has also been purified from avian and beef liver (Utter and Keech, 1960) and found to exhibit similar cofactor requirements to the oxaloacetate-producing reaction described for Cl. kluyveri, except that the pyruvic carboxylase of animal origin appeared to have a requirement for acetyl-CoA. Animal pyruvic carboxylase was inhibited by avidin. A logical conclusion would be that the oxaloacetate-forming reaction of Cl. kluyveri (Stern, 1963) was catalyzed by a pyruvic carboxylase.

The CO_2 exchange with pyruvate described by Stern (1963) was found to require a thiol, Mn^{++} or Co^{++}, cocarboxylase, flavin adenine dinucleotide or flavin monocucleotide and CoA. This exchange was insensitive to avidin and resulted in no net disappearance of pyruvate. The CO_2 exchange of Cl. kluyveri was very similar to that of Micrococcus lactilytus at acid pH (McCormick et al., 1962a; Whiteley and McCormick, 1963). Probably Stern's preparation of Cl. kluyveri fixed CO_2 in a pyruvate carboxylase-like reaction and exchanged CO_2 with pyruvate via a mechanism associated with the conversion of pyruvate to acetate.

It is quite possible that Shuster and Lynen (1960) could have had both types of reactions occurring in their preparations of Cl. kluyveri, i.e. a pyruvate carboxylase-like reaction and a pyruvate decarboxylation reaction. If the pyruvic carboxylase-like reaction is reversible as indicated by Utter and Keech (1960), it would give the effect of CO_2 exchange with pyruvate. This reaction would be particularly important in the CO_2 exchange if a fractionation procedure happened to concentrate the pyruvic carboxylase-like enzyme to a greater extent than the pyruvic-decarboxylating enzyme. The inhibition of exchange exerted by avidin (Shuster and Lynen, 1960) could be due to an inhibition of pyruvic carboxylase, or a pyruvic carboxylase-like enzyme present in the preparation. Biotin would not necessarily be involved in the conversion of pyruvate to acetate as is suggested by Shuster and Lynen.

2. Cleavage of Succinate

It was demonstrated by Topper and Stetten (1954) that rat liver is capable of cleaving succinate to two acetate units. Seaman and Naschke (1956) also demonstrated a similar cleavage in tetrahymena. Wood, Stjernholm and Leaver (1956), studying the metabolism of succinate-2-C^{14} by P. arabinosum, found that the carbon atoms of the acetate formed contained equal amounts of radioactivity, indicating that central cleavage of succinate had not occurred.

It is concluded that pyruvate is the major glycolytic precursor of acetate, and that TPP is usually required as a cofactor for the conversion of pyruvate to acetate.

b. Routes of Propionate Production

1. The Lactate Route

In 1928, van Niel (cited by van Niel, 1953), having recognized the central role of glycolysis in fermentation and having observed that various bacteria were able to form propionic acid from a number of carbohydrates, pyruvate and lactate, postulated that propionic acid formation involved the reduction of pyruvic acid to lactic acid, from which a molecule of water was eliminated giving rise to acrylic acid. Acrylic acid was then considered to be reduced to propionic acid. This postulation was made strictly on the basis of known biochemical mechanisms and was not substantiated by experimental evidence.

Ladd (1959) demonstrated that the rumen organism LC is able to ferment lactate. Washed cell suspensions and cell-free extracts of LC produced CO_2 and H_2, as well as acetic and propionic acids when lactate was the substrate. When lactate-2-C^{14} was used as the fermentation substrate, the resultant propionic acid was labelled almost exclusively in the methylene position. One molecule of propionate could have been formed from one molecule of lactate because the propionate produced was of the same specific activity

as the lactate utilized. In further studies with cell-free extracts of LC, Ladd and Walker (1959) found that lactate and acrylate were fermented at the same rate to similar end products. These workers proposed the scheme given in Fig. 1 to represent lactate fermentation by LC.

The evidence from which the scheme was derived deserves some discussion. It was found that the ability of LC extracts to ferment lactate, or acrylate, was lost upon short-term (5 hr) dialysis. The ability of such dialyzed extracts to ferment either lactate or acrylate was restored upon addition of catalytic amounts of ATP, ADP, pyruvate, or acetyl phosphate. The fact that the same compounds stimulated the fermentation of both substrates was evidence that the "sparkers", in the authors' terminology, acted through a common compound, which was suggested to be acetyl-CoA, but represented only as acetyl-X in Fig. 1. The finding that ATP and ADP were effective as "sparkers" while adenosine monophosphate (AMP) was not, indicated that the common intermediate was a high energy compound. Acetyl-CoA could represent a common point for the "sparkers", because the extracts used were able to decarboxylate pyruvate, contained acetokinase and probably contained phosphotransacetylase. The high energy derivative was considered to be necessary for the activation of lactate and acrylate prior to their interconversion. Acrylate and lactate were assumed to be activated by the extracts, because addition of hydroxylamine to the reaction mixture produced the hydroxamate of lactate and what was considered to be the polymerized product of the unstable acrylate hydroxamate. Extended dialysis of the LC extracts resulted in a loss of fermentative ability which could not be restored, even upon the addition of "sparkers". When substrate quantities of lactate or acrylate were incubated with catalytic quantities of the complementary compound, the extensively dialyzed extracts catalyzed the production of H_2. The moles of H_2 produced under these conditions were equivalent to the moles of "sparker"

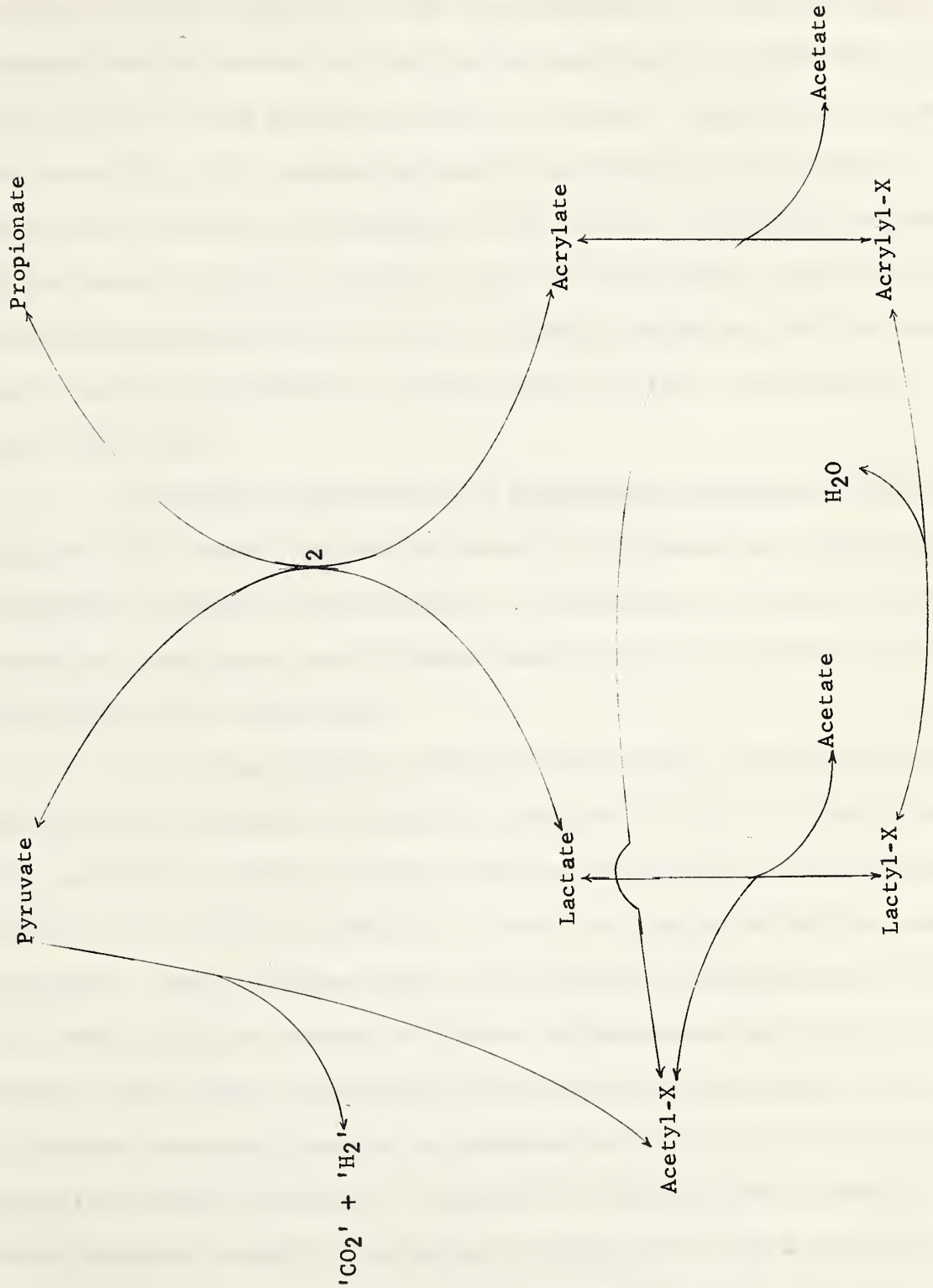

Figure 1. A pathway of propionate formation from lactate in the rumen organism LC (adapted from Ladd and Walker, 1959)

compound rather than to the moles of substrate compound. This was considered to be evidence that there was a coupled oxidation-reduction reaction involving lactate and acrylate as shown in Fig. 1, Reaction 2, page 9 . Further evidence for this coupling, and for the products of the coupled reaction was obtained when the extensively dialyzed extracts were incubated with an excess of lactate-2-C^{14} and substrate amounts of acrylate. Under these circumstances the moles of H_2, CO_2, acetate and propionate produced and the moles of acrylate and lactate utilized were all equivalent. The specific activity of the acetate produced was equal to that of the original lactate, while the propionate contained only a trace of activity, indicating that the lactate carbon had been converted to acetate and the acrylate to propionate as depicted in Fig. 1.

In studies using extracts of Clostridium propionicum, Stadtman and Vagelos (1957) showed that the CoA esters of propionate and acrylate were involved in propionic acid metabolism. These workers also found that extracts of a pseudomonad and of pigeon heart muscle were capable of converting acrylyl-CoA to lactyl-CoA.

If, in the studies of Ladd and Walker (1959), lactate was metabolized by the LC extracts in the manner depicted in Fig. 1, it would follow that equivalent amounts of acetate and propionate would be produced from lactate, if an influx of acetyl-X or acetyl-X-producing metabolites was prevented. Ladd and Walker (1959) found that when dialyzed extracts of LC were used, equivalent amounts of acetate and propionate were produced from lactate. The evidence presented by these workers would indicate that the LC extracts converted pyruvate to propionate entirely by way of the coupled oxidation-reduction reaction. This does not eliminate the possibility that other organisms, capable of producing propionate from lactate may do so, at least in part, by direct conversion of lactate to acrylate and acrylate to propionate.

2. The Succinate Route

i. Conversion of glycolysis intermediates to succinate precursors

In several bacteria, the lactate route of propionate formation can not account for all, or even any, of the propionic acid formed. Johns (1951b, 1952) stated that several strains of propionibacteria were unable to metabolize acrylate. Barker and Lipmann (1944) were able to eliminate the conversion of lactate to propionate by Propionibacterium pentosaceum when sodium fluoride was used as an inhibitor. Under these conditions the organism was still capable of converting pyruvate to propionate.

In 1948, Delwiche demonstrated that Propionibacterium pentosaceum was capable of decarboxylating succinic acid to propionic acid. Johns (1951a) demonstrated a similar conversion using Viellonella gazogenes. The decarboxylation of succinic acid would provide a route of propionate formation alternative to acrylate reduction. If glycolysis formed the central carbon supply route, a succinate decarboxylation pathway would necessitate the conversion of a glycolytic intermediate to a four carbon compound which in turn could be converted to succinate. A conversion of this type would be expected to involve a carbon condensation reaction. Wood et al. (1941) observed that the fermentation of glycerol by propionic acid bacteria involved CO_2 fixation. There could have been CO_2 condensation with a three carbon glycolysis intermediate to give rise to a four carbon succinate precursor. Thus far three conversions fitting this description have been proposed: the fixation of CO_2 with pyruvate giving rise to malate, with pyruvate giving rise to oxaloacetate and with phosphoenolpyruvate giving rise to oxaloacetate.

Ochoa, Mehler and Kornberg (1947, 1948) reported the partial purification of an enzyme from pigeon liver which catalyzed the reversible conversion of malate to pyruvate and CO_2 as in Reaction 3, Page 12.

3. 1-malic acid + TPN[1] $\rightleftharpoons$ pyruvic acid + CO_2 + TPNH[2]

The enzyme, called the malic enzyme, was also capable of catalyzing the reversible decarboxylation of oxaloacetate to pyruvate, and was specific for TPN, 1-malic acid and pyruvic acid. When the malic enzyme was coupled with an enzyme system such as glucose phosphate dehydrogenase to provide reduced pyridine nucleotide, it proved to be a very effective route of CO_2 fixation (Ochoa, Salles and Ortiz, 1950). It was found that the malic enzyme was induced in Lactobacillus arabinosus when the organism was grown in a medium containing l-malate (Blanchard et al., 1950). When the organism was adapted to malate in a biotin deficient medium, the malic enzyme activity was somewhat less than that resulting from adaptation on a biotin sufficient medium. Biotin would not restore the activity of the preparation from the deficient medium. Similarly, it was shown with turkeys (Ochoa et al., 1947) that malic enzyme activity of the tissues was decreased in biotin deficiency. Addition of biotin to the enzyme extract would not restore activity, and the enzyme could not be shown to contain biotin. It was therefore concluded that biotin was not a cofactor of the malic enzyme, but was concerned in the synthesis of the enzyme. A more recent study by Ables, Ravel and Shive (1961) has shown that biotin did stimulate malic enzyme synthesis in L. arabinosus grown on a low-biotin malic enzyme induction medium. The stimulatory effect of biotin could be replaced by L-asparagine, glycyl-L-asparagine or L-glutamyl-L-asparagine. Aspartate could not replace biotin under these conditions, but when the organism was grown in a biotin-free oleate medium, aspartate was effective. It was considered that L. arabinosus was unable to utilize exogenous aspartate under some conditions. The role of biotin was in the production of a four carbon compound necessary for aspartate synthesis. Hence, biotin is involved with the malic enzyme, but rather indirectly.

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[1] triphosphopyridine nucleotide (oxidized form)

[2] triphosphopyridine nucleotide (reduced form)

Utter and Keech (1960) have partially purified an enzyme from avian and beef liver which catalyzes the reaction:

4. pyruvate + ATP + CO_2 $\rightleftharpoons$ oxaloacetate + ADP + Pi .

It was found that Mg^{++} and a catalytic amount of acetyl-CoA were required. When acetyl-1-C^{14}-CoA was used no radioactivity was recovered in the oxaloacetate. This apparent requirement for acetyl-CoA was very similar to the results of studies carried out by Peel (1960), who found that the enzyme he was using appeared to have an absolute requirement for a catalytic amount of acetyl-CoA. It was later found that the enzyme preparation destroyed free CoA but not acetyl-CoA. The acetyl-CoA served only as a stable source of CoA for the enzyme system. The enzyme described by Utter and Keech (1960) was also found to be inhibited by avidin; an effect which was reversed by biotin.

Bloom and Johnson (1962), using a cell-free extract of Aspergillus niger, demonstrated the conversion of pyruvate to oxaloacetate as in Reaction 4. The carboxylation required Mg^{++} and K^{+}. No requirement for acetyl-CoA could be demonstrated. The inhibitory effect of avidin on the reaction was reversed by biotin. Similarly, Stern (1963) has reported reaction 4 in extracts of Clostridium kluyveri. In Cl. kluyveri also, the carboxylation of pyruvate to oxaloacetate was inhibited by avidin. The reaction was independent of cocarboxylase.

The conversion of phosphoenolpyruvate to oxaloacetate will be discussed as Reaction 5. This conversion can be accomplished by several different reactions. Utter and Kurahashi (1953, 1954) purified phosphoenolpyruvic carboxykinase from avian liver. The reaction catalyzed by this enzyme was as follows:

5a. phosphoenolpyruvate + CO_2 + CDP(IDP) $\rightleftharpoons$ oxaloacetate + CTP(ITP) .

Phosphoenolpyruvic carboxylase (Bandurski and Griener, 1953; Siu 1962a,b)

has been found in spinach. This enzyme catalyzes Reaction 5b.

5b. phosphoenolpyruvate + CO_2 + H_2O ⟶ oxaloacetate + Pi

Siu, Wood and Stjernholm (1961) and Siu and Wood (1962) have described an enzyme which they named phosphoenolpyruvic carboxytransphosphorylase. This enzyme was found in Propionibacterium shermanii and catalyzed Reaction 5c.

5c. phosphoenolpyruvate + CO_2 + Pi ⇌ oxaloacetate + PPi[1]

Phosphoenolpyruvic carboxytransphosphorylase was partially purified (Siu et al., 1961; Siu and Wood, 1962) and it was found that in addition to the reactants shown in Reaction 5c, Mg^{++} was required. Phosphoenolpyruvate could not be replaced by pyruvate. The reaction was reversible. Phosphoenolpyruvic carboxytransphorphorylase was not inhibited by preliminary incubation with avidin. P. shermanii was grown in a medium containing tritiated biotin and following this the phosphoenolpyruvic carboxytransphosphorylase purification procedure was carried out. It was found that as the enzyme was purified, the radioactive content per unit of protein decreased markedly, indicating that the enzyme and biotin were not associated.

In summation, it has been found that either of the glycolytic intermediates, phosphoenolpyruvate or pyruvate, can be carboxylated to one of the tricarboxylic acid cycle intermediates oxaloacetate or malate; the overall conversion being catalyzed by one or more of four enzymes.

ii. Conversion of oxaloacetate, malate and fumarate to succinate

It would be expected that oxaloacetate, malate and fumarate would be converted to succinate by means of the reversal of the well-known tricarboxylic acid cycle reactions catalyzed by malic dehydrogenase, fumarase and succinic dehydrogenase. These reactions are referred to hereafter as Reactions 6, 7 and 8 respectively.

Krebs and Eggleston (1941) found that the fumarate and malate were

[1] inorganic pyrophosphate

readily reduced to succinate by Propionibacterium shermanii. They found a powerful fumarase in this organism. Johns (1951a) demonstrated that Viellonella gazogenes produced propionate from fumarate and malate, while Delwiche (1948) showed that Propionibacterium pentosaceum was also capable of metabolizing these intermediates.

Ajl, Hart and Werkman (1950) found that dialyzed extracts of Escherichia coli metabolized succinate with a slower rate of O_2 uptake than that observed during the metabolism of succinate by non-dialyzed extracts. Addition of biotin to the dialyzed extracts resulted in an increased rate of O_2 uptake. It was therefore concluded that biotin could be cofactor of succinic dehydrogenase. With succinate as the substrate, Olson et al. (1948) found that cardiac slices from biotin deficient ducks exhibited a decreased rate of O_2 uptake as compared to slices from ducks fed biotin. The addition of biotin to the deficient slices did not increase the rate of succinate metabolism. Intraperitoneal injection of biotin into the birds for several days prior to preparation of the slices resulted in a reversal of the deficiency effect. It would appear that biotin may be associated with succinic dehydrogenase in some fashion but it is not clear whether biotin is involved as a coenzyme, or in the synthesis of the enzyme.

iii. Conversion of succinate to propionate

As was previously mentioned, it was demonstrated that several species of propionic acid-producing bacteria converted succinate to propionate (Delwiche, 1948; Johns, 1951a,b). On the basis of isotope data, Werkman and Wood in 1942 (cited by Delwiche, 1948) suggested that a symmetrical dicarboxylic acid was decarboxylated to propionic acid. The overall decarboxylation of succinate to propionate will be referred to as Reaction 9.

Whiteley (1953 a,b,c) used cell-free extracts of Micrococcus lactyliticus to study the decarboxylation of succinate, and demonstrated

that ATP and CoA were necessary for the reaction. Succinyl-CoA and propionyl-CoA were formed in the reaction mixture. The moles of propionyl-CoA formed corresponded very closely to the moles of CO_2 produced in the decarboxylation of succinate. The reaction was reversible.

Whiteley (1953c) concluded that the majority of the succinyl-CoA involved in the decarboxylation was formed by means of a transphorase reaction with propionyl-CoA as shown in Reaction 9a.

9a. succinate + propionyl-CoA $\rightleftharpoons$ succinyl-CoA + propionate

This conclusion was substantiated by the fact that only catalytic amounts of CoA and ATP were required for the decarboxylation, indicating that these compounds were necessary in the formation of the initial succinyl-CoA, and the CoA was thereafter recycled. Those extracts which were depleted of ATP and CoA were able to ferment succinyl-CoA, but not succinate plus CoA. Addition of succinate plus a small quantity of propionyl-CoA to depleted extracts resulted in a rapid evolution of gas, demonstrating that succinyl-CoA had been formed via a transphorase reaction and the CoA was used over in a cyclic manner. At this stage, the conversion of succinate to propionate was referred to by many workers merely as a decarboxylation, and very little was known of the enzymological aspects of the conversion. Whiteley (1953b) indicated that the overall reaction appeared to be most easily explained by a decarboxylation but that there could be a number of intermediate conversions involved.

The results of work on the metabolism of propionic acid by animal tissues suggested a number of possibilities for intermediate reactions in the decarboxylation of succinate to propionate. The metabolism of propionic acid was found to involve an initial ATP-dependent CO_2 condensation with propionyl-CoA to form methylmalonyl-CoA (Flavin and Ochoa, 1957) as in Fig. 2, Reaction 9b, page 17. Mazumder *et al.* (1962) have shown that the

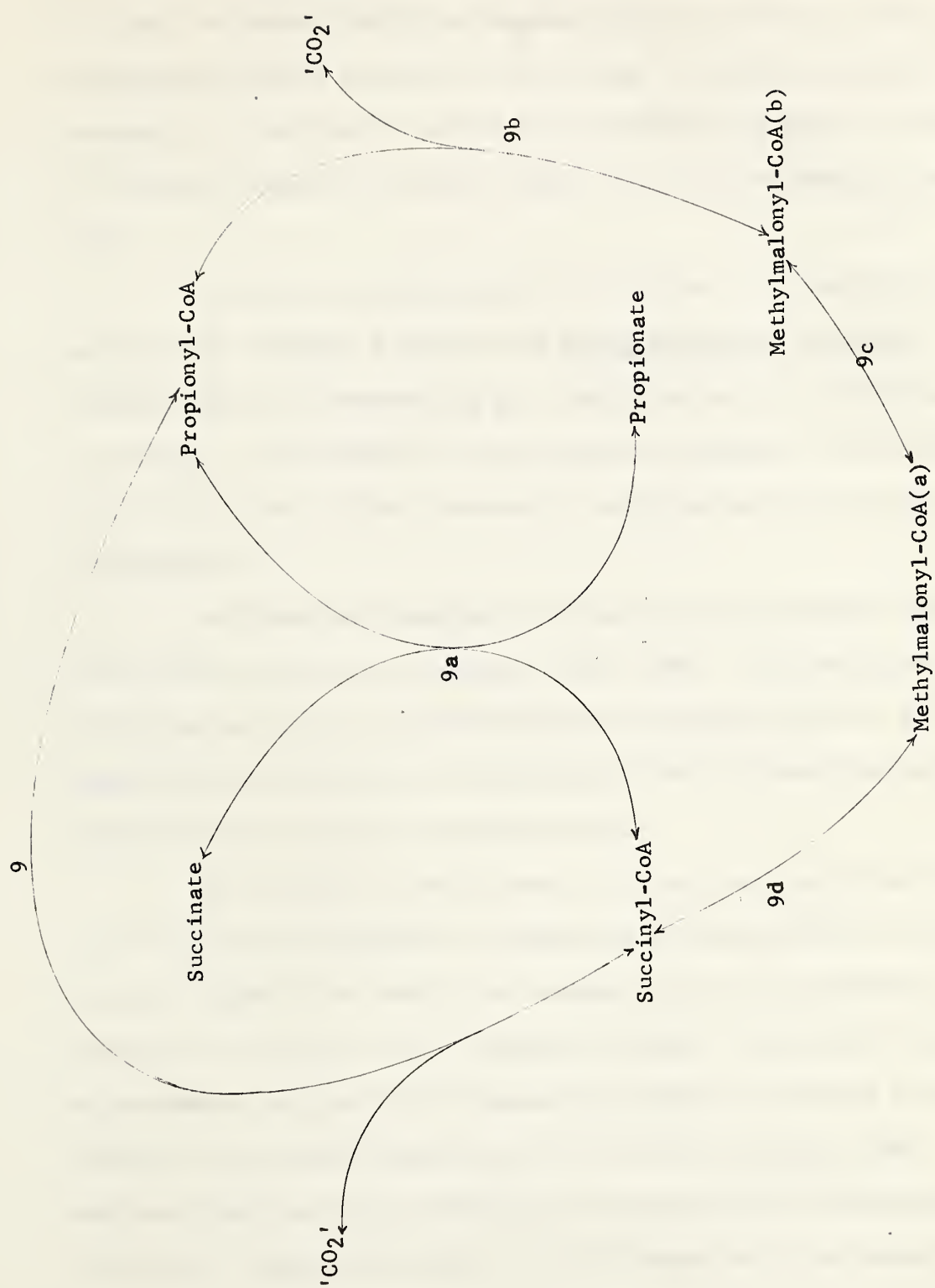

Figure 2. Possible routes for the bacterial decarboxylation of succinate to propionate

methylmalonyl-CoA is then racemized, as in Fig. 2, Reaction 9c, page 17, in a conversion catalyzed by methylmalonyl coenzyme A racemase. Mazumder, Sasakawa, and Ochoa (1963) have demonstrated the conversion of the racemized methylmalonyl-CoA to succinyl-CoA as in Fig. 2, Reaction 9d, page 17. This conversion is catalyzed by methylmalonyl coenzyme A mutase.[1] It was wondered if a similar series of reactions might be involved in propionic acid formation.

In 1961, Stjernholm and Wood succeeded in partially purifying methylmalonyl coenzyme A mutase from Propionibacterium shermanii. Like the mammalian enzyme of Mazumder et al. (1962) the bacterial mutase had a vitamin B_{12} coenzyme. The presence of this mutase in propionic acid bacteria suggests that succinyl-CoA is first converted to methylmalonyl-CoA during conversion to propionate.

Methylmalonyl coenzyme A racemase has been purified from propionic acid bacteria (Allen and co-workers, 1962, 1963). The mechanism of action of this enzyme has yet to be determined, but demonstration of its presence suggests the probability of racemization of methylmalonyl-CoA in the formation of propionic acid by propionibacteria.

On the basis of the enzymes that have been identified in propionic acid bacteria, the mechanism of conversion of succinyl-CoA to propionyl-CoA would appear to be simply the reverse of that for conversion of propionyl-CoA to succinyl-CoA in mammalian tissues. The initial step in the metabolism of propionyl-CoA in mammalian tissue is catalyzed by the enzyme propionyl carboxylase (Halenz et al., 1962; Kaziro et al., 1962). As mentioned previously the conversion of propionyl-CoA to methylmalonyl-CoA as in Fig. 2, Reaction 9b, page 17, is ATP-dependent. The reaction is actually a two-step conversion as follows:

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[1] In earlier literature this enzyme has been referred to as methylmalonyl coenzyme A isomerase.

$$\text{enzyme} + \text{ATP} + \text{"}HCO_3^-\text{"} \rightleftharpoons \text{ADP} + \text{Pi} + CO_2\text{-enzyme}$$

$$CO_2\text{-enzyme} + \text{propionyl-CoA} \rightleftharpoons \text{enzyme} + \text{methylmalonyl-CoA}$$

9b. sum: $\text{ATP} + \text{"}HCO_3^-\text{"} + \text{propionyl-CoA} \rightleftharpoons \text{ADP} + \text{Pi} + \text{methylmalonyl-CoA}$

Propionyl carboxylase of pig heart contained one mole of biotin per 150,000 g of protein and was inhibited by avidin (Kaziro, Leone and Ochoa, 1960) while propionyl carboxylase of bovine liver mitochondria contained one mole of biotin per 182,000 g of protein and was also inhibited by avidin (Halenz et al., 1962).

To the present there have been no reports of propionyl carboxylase of bacterial origin, but this does not eliminate the possibility of the occurrence of the conversion catalyzed by this enzyme in the formation of propionyl-CoA from succinyl-CoA, in bacteria. Assuming the existence of a reverse propionyl carboxylase reaction in bacteria and using the enzymes that have been found to be of propionic bacterial origin, the scheme of Fig. 2, page 17, represents a possible pathway of decarboxylation of succinate to propionate, although one must include the possibility of a succinate decarboxylation which does not involve methylmalonyl-CoA as an intermediate.

iv. The transcarboxylation pathway

All of the results obtained in studies of propionic acid formation by Propionibacteria spp. do not agree with the forementioned possible routes of propionate formation. Johns (1951b) observed that Propionibacteria spp. produced CO_2 from succinate only very slowly. Delwiche, Phares and Carson (1953, 1954) carried out studies on propionate formation using cell-free extracts of P. pentosaceum and found that CO_2 was produced from succinate only very slowly. Fermentation of succinate-1-C^{14} resulted in recovery of 30 per cent of the C^{14} as propionate-1-C^{14} and only 1.5 per cent as $C^{14}O_2$. Similarly, incubation of unlabeled succinate, propionate-2-C^{14}

and $C^{14}O_2$ gave rise to succinate-1,2-C^{14}, which was labeled in such a manner as to indicate that the propionate was exchanged 50 to 75 times more rapidly than the CO_2. From these observations the authors suggested that in the formation of propionate by P. pentosaccum, succinate was decarboxylated to a "C_1" compound which was not readily equilibrated with CO_2.

Leaver and Wood (1953) and Wood and Leaver (1953) carried out studies on the formation of propionate from pyruvate or glycerol. It was reasoned that, if the pathway involving CO_2 fixation with a three-carbon substrate followed by formation of succinate and subsequent decarboxylation of the succinate was actually operative in the propionibacteria, one mole of CO_2 should be fixed and one released for every mole of propionate produced. Thus, if $C^{14}O_2$ were used in the medium, 100 moles of labeled CO_2 should be fixed for every 100 moles of triose converted to succinate. The succinate so formed could be decarboxylated at either end. Hence, the overall effect would be a net fixation of 50 moles of $C^{14}O_2$ per 100 moles of propionate formed. However, it was found that upon fermentation of glycerol or pyruvate, there was a net fixation of only approximately 10 moles of $C^{14}O_2$ per 100 moles of propionate formed. This led to the conclusion that the major portion of the propionate was not formed by decarboxylation of succinate with formation of free CO_2.

Swick and Wood (1960) have proposed a transcarboxylation reaction to account for the conversion of methylmalonyl-CoA to propionyl-CoA. The "C_1" unit produced was not equilibrated with CO_2 but was postulated to be utilized in the coupled formation of oxaloacetate from pyruvate. Swick and Wood (1960) suggested that the overall transcarboxylation was the sum of the two coupled reactions, as shown below:

$$\text{methylmalonyl-CoA} + \text{enzyme} \rightleftharpoons \text{propionyl-CoA} + \text{enzyme-}C_1$$

$$\text{enzyme-}C_1 + \text{pyruvate} \rightleftharpoons \text{oxaloacetate} + \text{enzyme}$$

10. sum: methylmalonyl-CoA + pyruvate $\rightleftharpoons$ oxaloacetate + propionyl-CoA

Using cell-free extracts of P. shermanii Swick and Wood (1960) demonstrated that incubation of propionyl-C^{14}-CoA with oxaloacetate resulted in the formation of a labeled dicarboxylic acid. Malonate did not inhibit this reaction, indicating that the oxaloacetate was not oxidized to succinate. Succinate, malate, formate, isocitrate and α-ketoglutarate were ineffective as substitutes for oxaloacetate. The final products of the reaction were identified as succinyl-CoA (resulting from the isomerization of methylmalonyl-CoA to succinyl-CoA) and pyruvate. When pyruvate and succinyl-1,4,-C^{14}-CoA were incubated with the extracts, all of the radioactivity of the carboxyl groups of the resultant oxaloacetate was contained in the β-carboxyl group, indicating that the succinyl-CoA had been converted to methylmalonyl-CoA prior to transcarboxylation. Simple oxidation of the succinyl-CoA to oxaloacetate would have given activity in both carboxyl groups. Incubation of propionyl-CoA and oxaloacetate-4-C^{14} with the extract resulted in the more rapid formation of labeled methylmalonate than of labeled succinate. Also, in the presence of the extract, pyruvate plus methylmalonyl-CoA gave rise to much more rapid oxaloacetate formation than that observed from pyruvate plus succinyl-CoA. Thus, methylmalonyl-CoA, rather than succinyl-CoA, was considered to be involved in the reaction. The transcarboxylation was found to be inhibited by avidin; the inhibition being reversed upon addition of biotin. In a later study the transcarboxylase enzyme was partially purified from P. shermanii (Wood and Stjernholm, 1961). The stoichiometry of the reaction was shown to be as in Reaction 10, above. The enzyme was named methylmalonyl-oxaloacetic transcarboxylase. It was found that this enzyme was rather nonspecific for the CoA ester, being active with propionyl-CoA, acetyl-CoA, butyryl-CoA and acetoacetyl-CoA. The keto acid requirement was quite specific as none of the

group, α-ketobutrate, α-ketovalerate, α-ketoglutarate or β-ketoglutarate would substitute for pyruvate. The purified enzyme was inhibited by avidin, suggesting that biotin was a cofactor.

P. shermanii was grown in a medium containing tritiated biotin and methylmalonyl-oxaloacetic transcarboxylase was purified from the cells (Stjernholm et al., 1962; Wood et al., 1963). The enzyme contained 1.5 µg biotin per mg protein.

This enzyme could be very significant in explaining some of the previously observed anomalies in propionic acid fermentation. This would explain why the observed CO_2 turnover in the production of propionate from triose by propionibacteria was very low, because the 'C_1' of the transcarboxylation would not need to be interconverted with CO_2. Transcarboxylation would bring about the conversion of methylmalonyl-CoA to propionyl-CoA, independent of a reverse propionyl carboxylase-like step.

The scheme of propionate formation from pyruvate involving transcarboxylation as postulated by Swick and Wood (1960) is illustrated in Fig. 3. It is seen that the major reactions, excluding transcarboxylation, of this scheme have previously been discussed. The net effect of the scheme is the conversion of pyruvate to oxaloacetate, followed by reduction to succinate, which is converted to propionate. In these overall conversions, the transcarboxylation pathway as shown in Fig. 3 is not unique, but does avoid the problem of CO_2 fixation and release.

In summary there are two major routes of propionate formation from glycolytic intermediates; one involving lactic acid, the other succinic acid as an intermediate. The glycolytic precursor of the lactate route is pyruvate. There are a number of alternative conversions that could be involved in the overall succinate route. It can not be stated whether phosphoenolpyruvate or pyruvate is the immediate glycolytic precursor for the

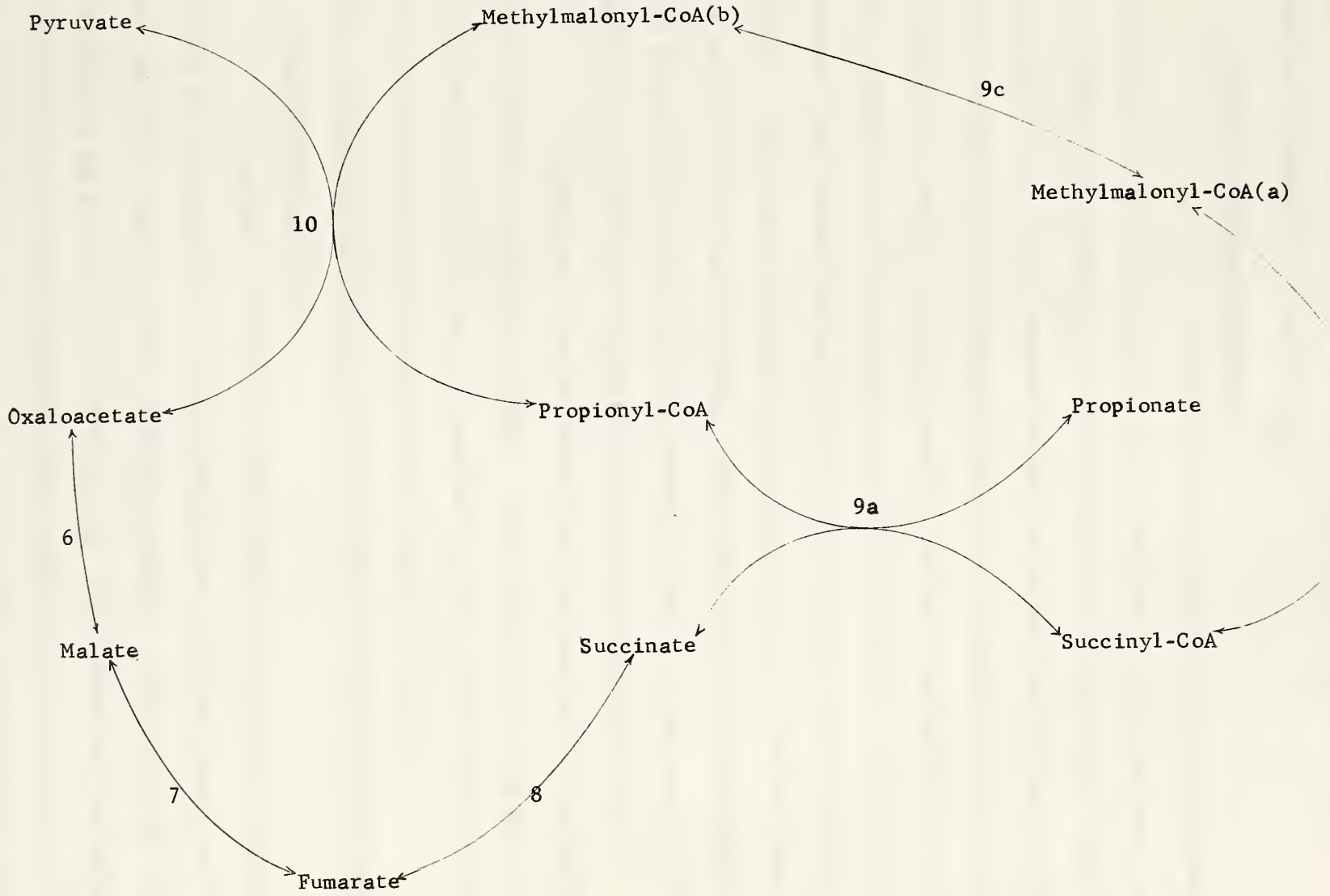


Figure 3. A pathway from pyruvate to propionate involving transcarboxylation

succinate route, as both could potentially be converted to a tricarboxylic acid cycle intermediate. The lactate route involves a rather direct reduction of pyruvate to propionate.

c. Route of Butyrate Production

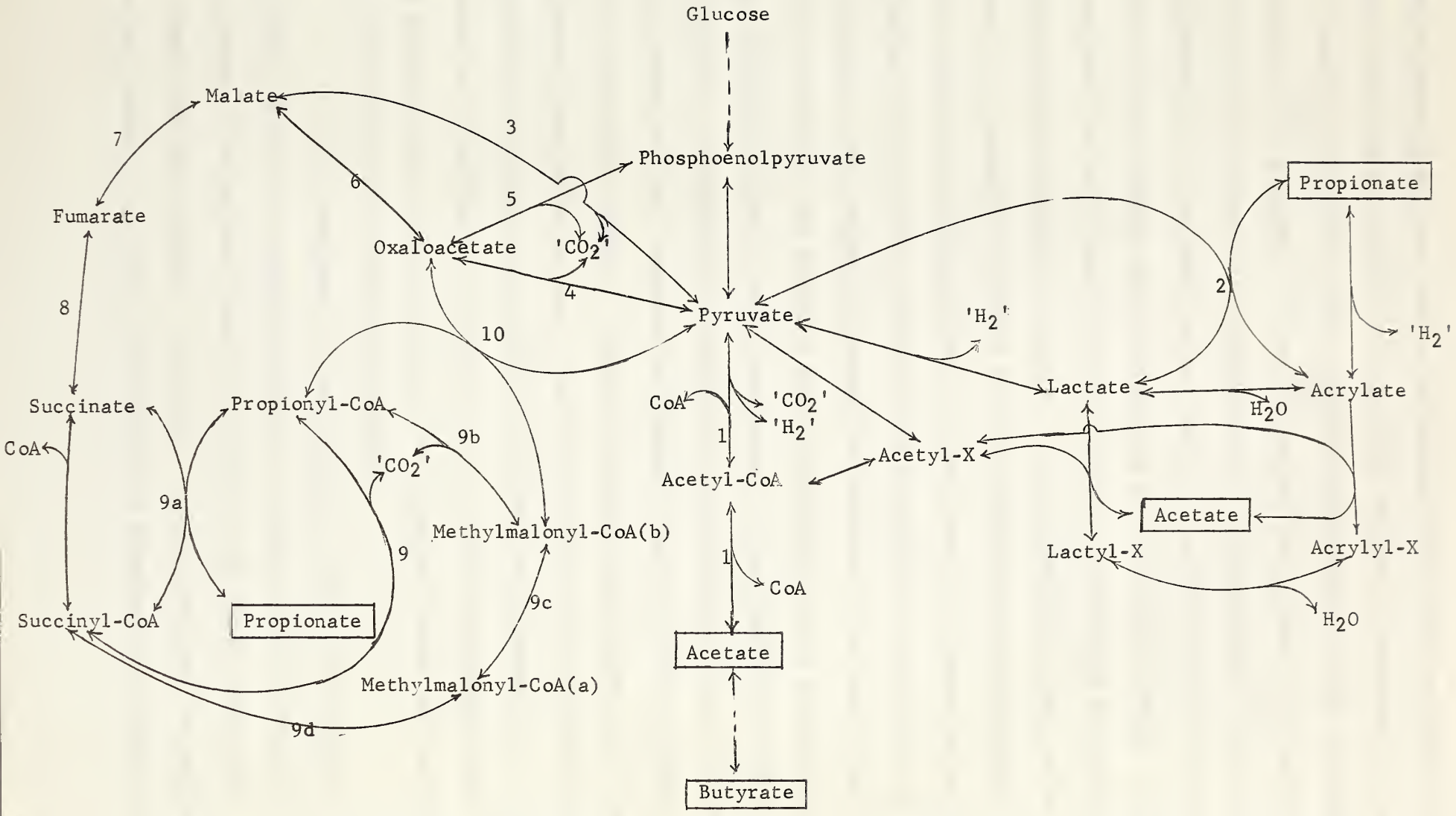
In comparison with the work that has been carried out on the formation of acetic and propionic acids, relatively little has been done to elucidate the pathway(s) for the formation of butyric acid. Elsden and Lewis (1953) found that butyrate as well as acetate and CO_2 were produced in the fermentation of pyruvate by washed cell suspensions of the rumen organism LC. Ladd (1959), while studying lactate fermentation with washed cell suspensions of LC found that the specific activity of the butyric acid produced upon fermentation of lactate-2-C^{14} was twice that of the substrate lactate. The acetate produced in this fermentation was labeled almost exclusively in the carboxyl group. Fifty per cent of the radioactivity of the butyrate was recovered in the carboxyl carbon. It was concluded that butyrate was formed via the condensation of two acetate molecules.

In summation it may be stated that the pure culture and enzyme work discussed is extremely valuable. When the various routes are combined, as in Fig. 4, a "map" is provided for the guidance and interpretation of studies of VFA formation by mixed bacterial cultures.

III. Fermentation of Potential VFA Precursors by Mixed Bacterial Populations

a. Conversion to VFA

Sijpesteijn and Elsden (1952) found that glucose was fermented by washed cell suspensions of rumen microorganisms with the production of CO_2 and one mole of acid per mole of hexose fermented. Doetsch et al. (1953), Hershberger et al. (1956) and Hueter et al. (1958) observed that fermentation of glucose by rumen microorganisms resulted in the production of acetic, propionic and butyric acids. Hueter et al. (1958) added glucose to the



Succinate route of propionate formation

Lactate route of propionate formation

Figure 4. Potential pathways of VFA formation

bovine rumen *in vivo* and observed a decrease in the molar per cent of acetic acid in the rumen fluid with a substantial increase in the molar per cent of propionic and butyric acids. Baldwin, Wood and Emery (1963) incubated glucose-1-C^{14}, glucose-6-C^{14} and glucose-2-C^{14} with rumen fluid and found that the acetate produced from glucose-1-C^{14} and glucose-6-C^{14} was labeled predominantly in the methyl carbon, while the acetate produced from glucose-2-C^{14} was labeled nearly exclusively in the carboxyl carbon. It was concluded that the glucose had been degraded via glycolysis to pyruvate which had been converted to acetate. The radioactivity of each carbon atom of the propionate produced from the labeled glucose was measured. It was found that the microorganisms from the rumen of an animal fed an all-roughage ration entirely randomized the radioactivity in C_2 and C_3 of propionate. This randomization would be expected if the glucose was converted to propionate via the succinate route; conversion via the lactate route would not randomize C_2 and C_3 of the propionate. The microorganisms from an animal fed a predominantly concentrate ration converted labeled glucose to propionate with the randomization between C_2 and C_3 of the propionate being only 70 per cent complete. This would indicate that 30 per cent of the glucose converted to propionate was converted via a non-randomizing route (perhaps the lactate route), while 70 per cent was converted via a randomizing route (perhaps the succinate route). It was consistently found that a greater portion of the activity of the substrate glucose was recovered as acetate than was recovered as propionate.

In the experiment of Doetsch *et al.* (1953) and Hershberger *et al.* (1956) pyruvate was converted primarily to acetic acid by rumen microorganisms although small amounts of propionic and butyric acids were formed from this compound. Pazur *et al.* (1958) found that the addition of a small amount of pyruvate-1-C^{14} during the fermentation of xylose by

suspensions of rumen bacteria resulted in recovery of the largest portion of the radioactivity as CO_2 and propionate. The acetate and butyrate were labeled only slightly. In this same study, incubation of pyruvate-3-C^{14} resulted in the recovery of the major portion of the radioactivity as acetate and butyrate. The acetate contained activity exclusively in the methyl position, and the butyrate activity was nearly entirely in the α- and γ-carbons and was equally distributed between the two. The radioactivity recovered in the propionate was found largely in the methyl carbon, although there had been some randomization to the methylene carbon. It was concluded that the mixed population decarboxylated pyruvate to acetate, two molecules of which condensed to form butyrate. Some pyruvate was converted to propionate and the majority of this conversion was accomplished via the lactate rather than the succinate route.

In 1956, Heuter, Shaw and Doetsch reported that incubation of sodium lactate with rumen ingesta resulted in an increase in the molar per cent of propionic and butyric acids and a decrease in the molar per cent of acetic acid. Bruno and Moore (1962) incubated uniform lactate-C^{14} with rumen ingesta and found that the final acetic acid contained about three times as much radioactivity as was recovered in propionic acid. The activity of the butyrate was slightly less than that of the propionate. It was therefore concluded that the majority of the lactate was converted to acetate. Jayasuriya and Hungate (1959) fermented lactate with rumen ingesta taken from cattle fed a number of different rations. The rations ranged in composition from predominantly concentrates to predominantly roughage. The lactate disappearance rate constant was 0.03 per min for the animals fed hay and 0.4 per min for the animals fed grain. The lactate pool size was determined to be a maximum of 0.0011 per cent (weight/volume) of the rumen ingesta. Thus on the basis of these data it was estimated that daily lactate conversion

could have been only equivalent to from one-thirteenth to one-sixth of the total substrate conversion in the rumen of animals fed grain, while the lactate conversion of animals fed hay was considerably lower. It was also found that the added lactate was converted predominantly to acetate, although it was also converted to propionate and butyrate.

Baldwin, Wood and Emery (1962) incubated lactate-2-C^{14} and lactate-3-C^{14} with rumen fluid from animals on rations of varying levels of concentrates. After incubation, the radioactivity of each of the carbon atoms of the propionic acid was determined. From the extent of randomization of activity between C_2 and C_3 of the propionate, it was calculated that 70 to 90 per cent of the lactate converted to propionate was converted via acrylate. These figures were later corrected to 54 to 88 per cent via the acrylate pathway (Baldwin et al., 1963). The concentrate level of the ration influenced the amount of lactate converted to propionate via acrylate versus succinate; there being a greater conversion via acrylate by organisms taken from an animal fed a predominantly concentrate ration. It is evident that mixed rumen populations are able to metabolize lactate, and to produce acetic, propionic and butyric acids as end products of this metabolism.

Very little work has been done on the metabolism of oxaloacetate, malate and fumarate by the mixed rumen population. Doetsch et al. (1953) reported that fermentation of malic acid by rumen microorganisms resulted in the production of a predominance of acetic acid, while fumaric acid was converted to acetic acid only. Oxaloacetic acid was not metabolized.

When Sijpesteijn and Elsden (1952) added succinic acid to a sheep's rumen in vivo it was observed that only the concentration of propionic acid was increased. Hueter et al. (1958) reported that addition of sodium succinate to the bovine rumen in vivo resulted in a decrease in the molar per cent

of butyric acid, a large increase in the molar per cent of propionic acid and an increase in the total VFA concentration. Elsden and Sijpesteijn (1950), Johns (1951c) and Sijpesteijn and Elsden (1952) have reported that washed suspensions of rumen bacteria converted succinate quantitatively to CO_2 and propionic acid. Blackburn and Hungate (1963) have carried out studies on the metabolism of succinate-2-3-C^{14} incubated briefly *in vitro* with bovine rumen digesta. They found that 75 per cent of the added succinate was converted to propionate with negligible conversion to butyrate or acetate. Approximately 40 per cent of the propionate formed was determined to have arisen from the succinate, but this was considered a minimal estimate. The rate of conversion of succinate to propionate was the same as the rate of formation of propionate. They therefore concluded that succinate was the major precursor of propionate in these studies.

Pazur *et al*. (1958) added a small amount of succinate-1-C^{14} to the medium when xylose was fermented by washed rumen organisms. The majority of the radioactivity was recovered as carbon dioxide and propionate, the propionate being labeled nearly exclusively in the carboxyl carbon.

Mixed rumen populations are quite capable of converting succinic acid to propionic acid.

b. Effects of VFA Precursors on Cellulose Digestion

Hershberger *et al*. (1956) carried out a series of experiments in which they incubated rumen bacteria with purified cellulose as the reference substrate. They added various intermediates of the scheme of Fig. 4 to the reaction mixture, and observed the effects not only upon VFA production (previously discussed), but also upon cellulose digestion. It was found that 0.02 M lactate depressed the cellulose digestion normally supported by a basal medium containing one gram of cellulose and 0.55 m moles of glucose

per 100 ml. At a similar concentration malate, glucose, pyruvate or succinate did not affect cellulose digestion. Dow (cited by Barnett and Reid, 1961) has also observed that lactate decreased cellulose digestion.

EXPERIMENTS AT THE UNIVERSITY OF ALBERTA

The experiments listed below were conducted during 1962 and 1963.

Experiment I — Effects of some metabolic antagonists and vitamin deficiencies upon cellulose digestion and VFA production.

Experiment II — Rate of cellulose digestion and the relationship of acetate, propionate and butyrate production to cellulose digested.

Experiment III — Effects of some metabolic intermediates on cellulose digestion in the presence of added biotin and in biotin deficiency and the conversion of the intermediates to volatile fatty acids in the presence of added biotin and in biotin deficiency.

Experimental

Preparation of Inoculum

Approximately 2 gallons of rumen ingesta were removed from a rumen fistulated Jersey cow that had been maintained on hay. The material was placed in three layers of cheese cloth and the liquid expressed with the aid of a mechanical press. As described by Johnson, Dehority and Bentley (1958), six pounds of the pressed rumen pulp were extracted with 1500 ml of phosphate buffer, made up with 1.059 g Na_2HPO_4 and 0.436 g KH_2PO_4 per liter. This buffer was saturated with CO_2 by rapidly bubbling the gas through the solution for 30 min. The solution was kept at 43 C during this saturation period. The buffer was transported to the farm, and the phosphate buffer extract (PBE) of the pressed rumen pulp was transported from the farm in two large vacuum flasks. The PBE was centrifuged, using a Sharples supercentrifuge, at approximately 30,000 rev/min and the sediment more than one inch above the bottom of the celluloid liner was resuspended, with the aid of a MSE Nelco 10 blender, in 175 ml of CO_2-saturated phosphate buffer at 40 C. This constituted the washed inoculum. To prepare a rewashed inoculum the sediment from the initial centrifugation was resuspended in 1500 ml of CO_2-saturated phosphate buffer rather than 175 ml of buffer. This suspension was centrifuged at 30,000 rev/min as before and the sediment more than one inch above the bottom of the celluloid liner was resuspended in 175 ml of CO_2-saturated phosphate buffer at 40 C to constitute the rewashed inoculum.

Preparation of Fermentation Tubes

The cellulose substrate used in all of the studies reported in this thesis was Solka Floc SW40A of which 200 $\pm$ 5 mg were weighed, air-dry, into each fermentation tube (90 ml Pyrex No. 8260). At the time the Solka

Floc was weighed into the fermentation tubes, three samples were taken for moisture analysis.

Either 5 ml of additive solution or 5 ml of distilled water were added to each tube prior to addition of the medium-inoculum mixture.

The components of the basal medium (Donefer, Crampton and Lloyd, 1960) were added to a 2 liter Erlenmeyer flask in quantities sufficient for 35 tubes. Sufficient distilled water was added to bring the volume to 1400 ml. This mixture was maintained at 40 C for 30 min while CO_2 was vigorously bubbled through the solution. At the end of this period 175 ml of inoculum suspension were added. The flask was placed on a magnetic stirrer and attached to an automatic pipette.[1] The fermentation tubes containing the cellulose plus 5 ml of additive or distilled water were placed in a water bath at 40 C. Into each tube were dispensed 45 ml of inoculum-medium mixture.

Fermentation Procedure

The general procedure of *in vitro* fermentation used was that described by Donefer *et al*. (1960), with the exceptions that the *in vitro* system consisted of 24 fermentation tubes through which CO_2 was bubbled at a rate of 60 to 120 bubbles per minute.

In each run, except where otherwise noted, 8 treatments, including the controls, each with 3 tubes per treatment, were used.

When rewashed inocula were employed, a fermentation period of 45 hr was used, while a fermentation period of 30 hr was used for the runs using washed inocula.

Upon conclusion of the fermentation, any material adhering to the CO_2 inlet or sides of the tube was washed into the tube with distilled water. The tube was removed from the water bath and the volume made up to 55 ml

[1]Model 40, Baltimore Biological Laboratory, Baltimore, Md.

with distilled water. The contents of the tube were mixed thoroughly and the insoluble material sedimented at 2,200 rev/min for 8 min in a refrigerated centrifuge[1] at 0 C. Following centrifugation, a 2 ml sample of the supernatant of each tube was transferred to an appropriately identified 2 ml plastic vial and the vial was sealed with a plastic cap. The vials and contents were stored at -15 C until used for VFA analysis. The remaining supernatant fluid was discarded.

Cellulose Determination

The cellulose content of the cake of insoluble material left in the fermentation tube after centrifugation was determined by a modification of the method of Crampton and Maynard (1938). Twenty ml of 80 per cent acetic acid, and 2 ml of concentrated nitric acid were added to the residue in each tube. A glass stirring rod was inserted into each tube, the contents thoroughly mixed and the stirring rod left in the tube. The tubes were placed in boiling water and stirred at 10 min intervals. At the end of 30 min the tubes were removed from the boiling water bath and cooled for 5 min. Twenty-five ml of 95 per cent ethanol were added to each tube. The tube contents were then quantitatively transferred to Selas crucibles of extremely coarse porosity using 95 per cent ethanol as the wash solution. The material retained in the crucible was washed with approximately 5 ml of acetone. The filtrate was discarded. The crucibles were then dried at 110 C, weighed, ashed at 550-600 C and re-weighed. The loss of weight upon ashing was considered equivalent to the weight of residual cellulose. The difference between the moisture-free weight of cellulose added to a tube and the weight of residual cellulose was considered to be the weight of cellulose digested in that tube.

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[1]MSE "Major" Centrifuge, Measuring and Scientific Equipment Ltd., Spenser Street, London, England.

VFA Analysis

MacKay (1963), working in this laboratory, developed a gas-liquid chromatographic method for the direct quantitative estimation of VFA in rumen fluid. With slight modification this method was found to be applicable to the estimation of VFA in the supernatant from artificial rumen fermentations. The instrument[1] used was equipped with a flame ionization detector. The column, a 2.5 m x 3 mm internal diameter stainless steel tube, was packed with 10% (weight/weight) 12-hydroxystearic acid on Fluoropak 80[2]. It was found that Teflon 6[3] could not be used in place of Fluoropak 80. The column temperature was maintained at 175 C with the flash vaporizer temperature 5 C below, and the detector temperature 50 C above, that of the column. The flow rate of the carrier gas (He) was maintained at 55 ml/min. Hydrogen and air flows to the detector were maintained at 20 ml/min and 325 ml/min respectively. The collector plate potential was kept at 250 V. The electrometer signal was fed to a potentiometer recorder[4] with a full scale deflection at 1 mv and pen speed of 1 sec.

Before analysis, the 2 ml supernatant samples were acidified with 0.03 ml conc. HCl. It was found that H_3PO_4 and H_2SO_4 were unsuitable as these acids produced considerable interference with the chromatograph.

The acidified solution was thoroughly mixed and from this solution approximately 9 µl were withdrawn with a fixed-needle syringe[5]. The volume of solution injected into the instrument ranged from 3 to 7 µl, depending upon the stability of the baseline of the instrument that day.

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[1]Burrell Kromo-Tog, Ionization Model K-7, Burrell Corporation, Pittsburg, Pa.
[2]The Fluorocarbon Co., 1206 East Ash Ave., Fullerton, Calif.
[3]Analytical Engineering Laboratories Inc., Handen 18, Conn.
[4]Model Y143x58-UB-11-111-30V, Minneapolis-Honeywell Regulation Co., Brown Instruments Division, Philadelphia, Pa.
[5]Number 701-N, Hamilton Co., Inc., Whittier, Calif.

The fatty acids were eluted from the column in the order acetic, propionic, iso-butyric, n-butyric, iso-valeric and n-valeric in 20 min following injection. Iso-butyric acid was not entirely separated from n-butyric and propionic acids.

Five standard solutions of the VFA in distilled water were prepared. A wide range of concentrations of each of the fatty acids was contained in the standard solutions. A different standard solution was injected after every 4 unknown samples. The entire range of standards was analyzed at least once a day. A calibration curve was drawn for each acid for each day. In order to prepare a calibration curve for any individual acid, the weight, corrected for sample size, of the chart paper under the curve was plotted against the concentration of that acid in the standard from which the curve was obtained. Unknown acid concentrations were then readily determined by fitting the corrected curve weight to the appropriate calibration curve. All concentrations were expressed as mmoles/100 ml.

Thus, by using daily calibration curves, the instrument conditions and injection-sample volume could be changed from day to day, as was often found necessary, while the final VFA estimates remained quantitative.

All the fermentation samples analyzed contained a trace of iso-butyric acid, but that amount was too small to be estimated; iso-valeric and valeric estimates were subject to considerable variability; the amount of iso-valeric acid present was always very small and it was never demonstrated that the valeric acid content of the fermentation mixtures differed from that of the medium. Therefore, in this thesis, only the acetic, propionic and butyric acid estimates are discussed, and hereafter the term volatile fatty acids (VFA) will be used to denote only these three acids.

Statistical Analyses

The statistical methods used to aid in the interpretation of the data in this thesis have been described by Steel and Torrie (1960).

Experiment I

Effects of Some Metabolic Antagonists and Vitamin Deficiencies Upon Cellulose Digestion and VFA Production

Trial 1

Introduction and Object

Thiamine has been shown to be a coenzyme for the decarboxylation of pyruvate by several enzyme preparations. If acetate is produced in the rumen by the decarboxylation of pyruvate, it is quite likely that thiamine is involved. This trial was designed to determine the effects of the thiamine antagonists oxythiamine, neopyrithiamine, thiopental or hexetidine on cellulose digestion and VFA production by rumen organisms in vitro.

Experimental.

Oxythiamine[1], neopyrithiamine[2], and thiopental[3] were readily soluble in water, and were therefore added in aqueous solution to the fermentation tubes. Hexetidine[4] was immiscible with distilled water, but became miscible when the water was acidified to pH 2 with HCl. Hexetidine was therefore added to the fermentation tubes in a 0.02 M HCl solution.

The concentrations of antagonists used in the fermentation mixture were as follows:

Oxythiamine: 6×10^{-5}, 1.2×10^{-3}, 6×10^{-3}, 3×10^{-2}, 6×10^{-2}, 3×10^{-1}, and 4 mM.

Neopyrithiamine: 1×10^{-3}, 2×10^{-2}, 4×10^{-2}, 8×10^{-2}, 1.2×10^{-1}, 2.0×10^{-1} and 2 mM.

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[1]Oxythiamine HCl, Nutritional Biochemicals Corp., Cleveland 28, Ohio.
[2]Neopyrithiamine HBr, California Corporation for Biochemical Research, 3625 Medford Street, Los Angeles 63, Calif.
[3]Thiopental sodium, the monosodium salt of 5-ethyl-5-(1-methylbutyl) -2-thiobarbituric acid, Abbott Laboratories Ltd., Montreal, Que, donated by Dr. D.C. Secord, University of Alberta.
[4]W1435 hexetidine (1,3-bis-β-ethylhexyl-5-methyl-5-aminohexahydropyrimidine) donated by Warner-Lambert Research Institute, Morris Plaines, N.J., research affiliate of Warner-Chilcott Laboratories.

Thiopental: 7.2×10^{-4}, 7.2×10^{-3}, 1.8×10^{-2}, 3.6×10^{-2}, 5.4×10^{-2}, 7.2×10^{-2}, and 7.2×10^{-1} mM.

Hexetidine: 2.9×10^{-4}, 2.9×10^{-3}, 5.9×10^{-3}, 1.2×10^{-2}, 1.5×10^{-2}, 1.8×10^{-2}, 5.9×10^{-2}, 5.9×10^{-1}, and 1.5 mM.

Oxythiamine, at all but the 4mM concentration, was used in studies with rewashed inocula. Washed inocula were used in studies with 4mM oxythiamine, neopyrithiamine, thiopental, and hexetidine.

Results

Oxythiamine at concentrations up to 4mM did not influence cellulose digestion or the production of acetate, propionate or butyrate (Table 1). Similarly, neopyrithiamine at concentrations ranging from 1×10^{-3} mM to 2 mM did not affect cellulose digestion, acetate production or propionate production, but in run 26, butyrate production was increased (significant at $P < 0.05$) (Table 2).

Thiopental at the concentration 7.2×10^{-1} mM resulted in significantly ($P < 0.01$) less cellulose digestion than that observed in the control tubes (Table 3). At this level of thiopental the production of acetate, propionate and of butyrate was significantly less ($P < 0.05$, $P < 0.01$ and $P < 0.05$, respectively) than in the control tubes. At lower concentrations thiopental had no effect on cellulose digestion or VFA production.

Hexetidine at the concentration 5.9×10^{-2} in the fermentation mixture was a very effective inhibitor of digestion (Table 4). Although cellulose digestion was completely inhibited by this concentration of hexetidine, there was still production of VFA presumably from the glucose of the medium. Higher levels of hexetidine (1.5 mM, run 9) inhibited VFA production entirely. At a concentration of less than 2.9×10^{-3} mM in the fermentation mixture hexetidine did not appear to have any influence upon

TABLE 1

Effects of oxythiamine HCl on cellulose digestion and VFA production

		Concentration of OT HCl[1] in fermentation mixture mM	Cellulose digested mg	VFA concentration (mmole/100 ml) Acetate	Propionate	Butyrate
Run 4		0	177	1.43	1.60	0.16
		6×10^{-5}	186	1.64	1.77	0.20
		1.2×10^{-3}	182	1.46	1.62	0.17
		6×10^{-3}	178	1.51	1.70	0.17
Mean Squares	OT HCl (3 df)		40.0	0.0261	0.0178	0.00067
	Error (8 df)		12.3	0.0121	0.0134	0.00086
Run 5		0	187	1.90	1.55	0.18
		3×10^{-2}	183	1.89	1.50	0.16
		6×10^{-2}	184	1.96	1.55	0.16
		3×10^{-1}	185	1.88	1.49	0.17
Mean Squares	OT HCl (3 df)		8.22	0.00363	0.00363	0.00057
	Error (8 df)		3.29	0.0127	0.0199	0.00127
Run 26		0	145	2.00	1.46	0.15
		4	136	2.00	1.19	0.14
Mean Squares	OT HCl (2 df)		82.3	0	0.109	0.0002
	Error (3 df)		68.9	0.0952	0.0329	0.00048

[1] oxythiamine HCl

TABLE 2

Effects of neopyrithiamine HBr on cellulose digestion and VFA production

		Concentration of NPTHBr[1] in fermentation mixture mM	Cellulose digested mg	VFA concentration (mmole/100 ml) Acetate	Propionate	Butyrate
Run 22		0	148	1.68	1.51	0.23
		1.0×10^{-3}	138	1.61	1.48	0.23
		1.0×10^{-2}	145	1.72	1.51	0.23
		4.0×10^{-2}	137	1.65	1.49	0.22
		8.0×10^{-2}	143	1.66	1.47	0.25
		1.2×10^{-1}	146	1.64	1.51	0.25
		1.6×10^{-1}	147	1.65	1.40	0.25
		2.0×10^{-1}	143	1.65	1.48	0.24
Mean Square	NPTHBr (7 df)		49.3	0.00319	0.00383	0.00040
	Error (16 df)		26.5	0.0190	0.00586	0.00059
Run 26		0	145	2.00	1.46	0.15
		2	132	1.97	1.44	0.18
Mean Square	NPTHBr (1 df)		169	0.0011	0.0005	0.0008*
	Error (2 df)		50.3	0.0019	0.0063	0.00003

*significant at $P < 0.05$

[1]neopyrithiamine HBr

TABLE 3

Effects of thiopental sodium on cellulose digestion and VFA production

		Thiopental concentration in fermentation mixture mM	Cellulose digested mg	VFA concentration (mmole/100 ml) Acetate	Propionate	Butyrate
Run 13		0	177	2.55	1.81	0.41
		7.2×10^{-4}	171	2.35	1.69	0.38
		7.2×10^{-3}	175	2.44	1.74	0.40
		1.8×10^{-2}	171	2.33	1.75	0.38
		3.6×10^{-2}	173	2.38	1.70	0.42
		5.4×10^{-2}	174	2.40	1.73	0.38
		7.2×10^{-2}	174	2.40	1.74	0.37
		7.2×10^{-1}	145^{DD}	2.05^{D}	1.35^{DD}	0.32^{D}
Mean Squares	Thiopental (7 df)		316**	0.06041*	0.05977**	0.00271*
	Error (16 df)		6.59	0.01582	0.006375	0.00100

*significant at $P < 0.05$

**significant at $P < 0.01$

^{D}Mean which is significantly different from the control mean at $P < 0.05$ (Dunnett's method)

^{DD}Mean which is significantly different from the control mean at $P < 0.01$ (Dunnett's method)

TABLE 4

Effects of hexetidine on cellulose digestion and VFA production

		Hexetidine concentration in fermentation mixture mM	Cellulose digested mg	VFA concentration (mmole/100 ml) Acetate	Propionate	Butyrate
Run 11		0	179	1.86	1.55	0.41
		2.9×10^{-4}	174	1.73	1.41	0.39
		2.9×10^{-3}	169	1.80	1.54	0.36
		1.5×10^{-2}	72.2^{DD}	1.27^{DD}	0.97^{DD}	0.21^{DD}
Mean Squares	Hexetidine (3 df)		7,780**	0.214**	0.226**	0.0249**
	Error (8 df)		8.44	0.00489	0.00218	0.00064
Run 12		0	176	2.72	1.33	0.79
		5.9×10^{-3}	170	2.71	1.38	0.75
		1.2×10^{-2}	169	2.53	1.43^{D}	0.71^{D}
		1.8×10^{-2}	161^{D}	2.27	1.42^{D}	0.59^{DD}
Mean Squares	Hexetidine (3 df)		95.0*	0.104	0.00563**	0.0172**
	Error (7 df)		17.8	0.0433	0.00064	0.0015
Run 9		0	183	1.63	1.66	0.15
		5.9×10^{-2}	0	0.48	0.45	0.05
		5.9×10^{-1}	0	0.34	0	0
		1.5	0	trace	trace	0

* significant at $P < 0.05$
**significant at $P < 0.01$
^{D} mean which is significantly different from the control mean at $P < 0.05$ (Dunnett's method)
^{DD}mean which is significantly different from the control mean at $P < 0.01$ (Dunnett's method)

cellulose digestion or VFA production by rewashed bacterial suspensions.

At a concentration of 1.5×10^{-2} mM in the fermentation mixture, (in run 11) hexetidine resulted in statistically significant ($P < 0.01$) decreases in cellulose digestion, acetate, propionate and butyrate production (Table 4). The decrease in acetate production was of the same magnitude as the decrease in propionate production. When hexetidine was present in the fermentation mixture at a concentration of 1.8×10^{-2} mM, in run 12, there was a nonsignificant decrease in acetate production, a significant ($P < 0.01$) decrease in butyrate production and a significant ($P < 0.05$) increase in propionate production. It was found that the response to hexetidine varied greatly between runs. This variability becomes evident when the effect of 1.5×10^{-2} mM hexetidine on cellulose digestion in run 11 is compared to the effect of 1.8×10^{-2} mM hexetidine on cellulose digestion in run 12; the lower level in run 11 resulted in a much greater inhibition than did the higher level in run 12 (Table 4).

Discussion

Oxythiamine and neopyrithiamine are antagonists of thiamine in a number of animals, microorganisms, and enzyme systems (Woolley, 1952; Cerecedo, 1955; Rogers, 1962).

The fact that neither oxythiamine nor neopyrithiamine produced effects in this study could be attributable to one or more of the following possibilities. It is possible that thiamine is not required for any of the processes involved in the digestion of cellulose, or the production of VFA in the artificial rumen, although this would not be expected. Oxythiamine and neopyrithiamine are competitive antagonists of thiamine, and in order to observe an effect from these compounds it has consistently been found that the molar ratio of inhibitor to metabolite must be rather large. It is known that thiamine is synthesized by the rumen population, and would

presumably be synthesized by the population of the in vitro system. It is possible that even the highest levels of oxythiamine and neopyrithiamine were not sufficient to compete with the endogenous plus newly synthesized thiamine in the system. Scherr and Rafelson (1962) showed that in a culture of Escherichia coli incubated in a medium containing pyrithiamine, some cells developed the capacity to produce abnormally large amounts of thiamine. These cells grew quite readily in the presence of the antagonist. This could have occurred in the present study when oxythiamine and neopyrithiamine were added to the medium.

Hexetidine has been reported to inhibit dehydrogenase activity of human leucocytes (Lionetti, Telles and Avery, 1960), oxygen consumption of human erythrocytes (Lionetti, McLennan and Communale, 1959) and pyruvate oxidation in a particulate fraction of Bacillus cereus var. terminalis spores (Halvorson and Church, 1957). The latter two effects were at least partially reversed by TPP (Lionetti et al., 1959; Halvorson and Church, 1957). In run 11, it was shown that hexetidine would inhibit acetate production to the same extent as it inhibited propionate production. In run 12, on the other hand, it was found that hexetidine would decrease acetate production but increase propionate production. Thus the effect of hexetidine on fatty acid production would not appear to agree between the two runs. This discrepancy is probably a reflection of the differences in overall response to hexetidine in these runs. In run 11 there was a drastic inhibition of cellulose digestion which may have confounded any effects on fatty acid production. The results of hexetidine inhibition in run 12 suggest that this compound could have inhibited the function of thiamine in pyruvate decarboxylation.

Fahmy, Ibrahim and Talaat (1962) have reported that thiopental inhibited the oxidation of pyruvate in brain homogenates. This inhibition

was partially reversed by TPP and entirely reversed by the simultaneous use of TPP and lipoic acid. At a concentration of 7.2×10^{-1} mM in the fermentation mixture, thiopental inhibited acetate production as effectively as it did proprionate production. Butyrate is considered to be formed from the condensation of two acetate units. If the reduction of butyrate production in the presence of 7.2×10^{-1} mM thiopental was due to a lack of acetate it would be concluded that acetate production was decreased to a greater extent than propionate production by 7.2×10^{-1} mM thiopental. This effect would be expected if thiopental inhibited the conversion of pyruvate to acetate through an inhibition of the coenzyme function of thiamine.

The exact metabolic sites at which hexetidine and thiopental exert their inhibitory effects deserve further investigation.

Summary

1. Oxythiamine at concentrations ranging from 6.0×10^{-5} to 4.0 mM in fermentation mixture, and neopyrithiamine at concentrations ranging from 1.0×10^{-3} mM to 2 mM did not influence in vitro cellulose digestion, or VFA production by rumen microorganisms.

2. Both thiopental and hexetidine inhibited cellulose digestion.

3. Thiopental inhibited acetate and propionate production nearly equally. In one run it appeared that hexetidine could produce a greater inhibition of acetate production than of propionate production, although in another run hexetidine inhibited acetate and propionate production equally.

Trial 2

Introduction and Object

Biotin has been shown to be a cofactor of a number of enzymes which could be involved in the production of propionic acid. Propionic acid is one of the major end products of rumen metabolism. It was there-

fore decided to carry out a study of the effects of the biotin antagonists, desthiobiotin and avidin, upon the digestion of cellulose and production of VFA by rumen microorganisms in vitro.

Experimental

Prior to incubation, desthiobiotin[1] or avidin[1,2] was added to the fermentation tubes in aqueous solution, in quantities sufficient to result in the following concentrations in the fermentation mixture.

Desthiobiotin: 4.7×10^{-2}, 9.3×10^{-2} and 4.7×10^{-1} mM.

Avidin: 1.5, 7.0 and 12.0 units per tube.

Rewashed inocula were used in the studies with desthiobiotin, while washed inocula were used in the avidin studies.

Results

Over the range of concentrations used, desthiobiotin did not affect cellulose digestion or exert any consistent influence on the production of acetate, propionate, or butyrate (Table 5).

Avidin, at a level of 1.5 units per tube in run 14, had no effect on cellulose digestion or VFA production (Table 6). At 7.0 units per tube in run 14, avidin decreased cellulose digestion (significant at $P < 0.01$) but had no effect on VFA production. In run 15, 12 units of avidin per tube caused a drastic reduction in cellulose digestion (Table 6). This effect was partially reversed by the addition of 30 ug of biotin with the avidin. Twelve units of avidin caused a decrease in acetate production of 0.78 mmoles per 100 ml (significant at $P < 0.01$) and in propionate production of 1.15 mmoles per 100 ml (significant at $P < 0.01$). When 30 ug of biotin were added with 12 units of avidin there resulted an insignificant decrease in acetate ($p > 0.01$) and butyrate ($P < 0.05$) production and a decrease in propionate production of 0.38 mmoles per 100 ml which was significant at $P < 0.01$.

[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.
[2]2500 units per g.

TABLE 5

Effects of desthiobiotin on cellulose digestion and VFA production

		Desthiobiotin concentration in fermentation mixture mM	Cellulose digested mg	VFA concentration (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 5		0	186	1.90	1.55	0.18
		4.7×10^{-2}	187	1.89	1.50	0.16
		9.3×10^{-2}	189	1.96	1.55	0.16
		4.7×10^{-1}	186	1.88	1.49	0.17
Mean Squares	Desthiobiotin (3 df)		4.17	0.0393	0.00800	0.00040
	Error (11 df)		3.97	0.0191	0.0204	0.00084

TABLE 6

Effects of avidin on cellulose digestion and VFA production

		Avidin concentration units/tube	Cellulose digested mg	VFA concentration (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 14		0	180	1.81	1.87	0.22
		1.5	178	1.87	1.95	0.24
		7.0	172^{DD}	1.70	1.79	0.23
Mean Squares	Avidin (2 df)		55.3**	0.0222	0.0209	0.0003
	Error (6 df)		3.74	0.0120	0.00743	0.0001
Run 15		0	181^{c}	1.69^{b}	1.80^{c}	0.13^{y}
		12	45^{a}	0.91^{a}	0.65^{a}	0.08^{x}
		12 + 30 ug Biotin	143^{b}	1.32^{ab}	1.42^{b}	0.09^{xy}
Mean Squares	Avidin (2 df)		14,800**	0.449**	1.042**	0.0023*
	Error (6 df)		262	0.00858	0.00388	0.00042

*significant at $P < 0.05$
**significant at $P < 0.01$
^{D}mean which is significantly different from the control mean at $P < 0.05$ (Dunnett's Method)
^{DD}mean which is significantly different from the control mean at $P < 0.01$ (Dunnett's Method)
^{x,y}means in the same column not identified by the same superscript are significantly different at $P < 0.05$ (Duncan's multiple range test)
^{a,b,c}means in the same column not identified by the same superscript are significantly different at $P < 0.05$ (Duncan's multiple range test)

Discussion.

Woolley (1952) has discussed the work of other investigators which has shown that the effectiveness of desthiobiotin as a biotin antagonist varied greatly among microorganisms. With some organisms it was a potent antagonist, while with others, desthiobiotin could satisfy the biotin requirement. Thus, it would be expected that in the tremendously versatile, mixed inoculum of rumen microorganisms, there could be some organisms for which desthiobiotin could have been inhibitory, but others for which it may have been noninhibitory or even beneficial. The preferential growth of organisms able to tolerate or utilize desthiobiotin would result in a change in the bacterial population, but this would not necessarily influence any of the criteria observed in the present study.

Twelve units of avidin inhibited cellulose digestion and VFA production in run 15. It is not known if these effects resulted from an inhibition of biotin at different points or only a single metabolic point. Metabolism of the glucose units produced from cellulose hydrolysis results in the production of VFA. Therefore, an inhibition of cellulose digestion would be expected to result in an inhibition of VFA production. On the other hand, it has been established that biotin is a necessary cofactor for a number of the enzymes which could be involved in the production of propionate from succinate. If inhibition of one or more of these enzymes occurred, propionate production would be decreased. At the same time, the metabolic block would result in a build-up of intermediates which could inhibit cellulolysis. Those intermediates 'downstream' from the block would be depleted, and if these were required by cellulolytic microorganisms, cellulolysis would decrease. Decreased cellulose digestion would result in decreased VFA production. Therefore, the net effect of biotin inhibition would be an inhibition of cellulose digestion and VFA production.

It would be expected that the production of propionate would be inhibited to a greater extent than that of acetate, or butyrate if avidin produced an inhibition of the function of biotin in the succinate route of propionate formation. It was found that when 12 units of avidin were added to the fermentation tubes propionate production was inhibited to a greater extent than acetate production. Thus, the experimental results suggest that avidin inhibited the function of biotin at some point on a pathway of propionate production. That avidin was actually inhibiting the action of biotin was demonstrated by the reversal of avidin inhibition by biotin.

It would be valuable to determine the exact metabolic function of biotin that was inhibited by avidin in the artificial rumen system.

Summary

1. Desthiobiotin at concentrations from 4.7×10^{-2} to 4.7×10^{-1} mM in the in vitro fermentation mixture did not influence cellulose digestion or the production of acetate, propionate, or butyrate by rewashed suspensions of rumen microorganisms.

2. At a level of 1.5 units per tube, avidin did not influence cellulose digestion or VFA production, while 7.0 units of avidin decreased cellulose digestion, but did not influence VFA production.

3. Avidin, at a level of 12 units per tube resulted in a definite inhibition of cellulose digestion. Under these conditions propionate production was inhibited to a greater extent than acetate production, while there was only a slight inhibition of butyrate production.

Trial 3

Introduction and Object

The addition of avidin to the medium, in Trial 2, was found to result in a marked reduction of cellulose digestion and VFA production by rumen organisms incubated in vitro. It was therefore decided to carry out

studies to determine the effects of the deletion of biotin from the *in vitro* medium. Para-aminobenzoic acid (PABA) is routinely added to the *in vitro* medium. The effects of the removal of this vitamin from the *in vitro* medium were also of interest.

Experimental

Either the biotin or PABA solution was omitted from the medium in the treatment tubes and an equivalent volume of distilled water added in its place. PABA deletion was studied in runs employing rewashed inocula only, while biotin deletion was studied using both washed and rewashed inocula.

Results

The deletion of PABA from the fermentation medium resulted in a decrease in cellulose digestion of 11 mg per tube which was significant at $P < 0.01$ (Table 7). PABA deletion did not influence acetate production, but a decrease of 0.18 mmoles per 100 ml in propionate production was observed. This decrease was statistically significant ($P < 0.01$). There was a decrease (significant at $P < 0.05$) in butyrate production of 0.02 mmoles per 100 ml upon deletion of PABA.

The deletion of biotin from the fermentation medium resulted in a decrease in cellulose digestion of 113.3 mg per tube accompanied by significant ($P < 0.01$) decreases in acetate and propionate production of 0.56 and 1.00 mmoles per 100 ml respectively. There was not a significant effect upon butyrate production (Tables 8 and 9). The differences in magnitude of the effects on cellulose digestion and acetate and propionate production between runs were significant at $P < 0.01$. There was also a significant ($P < 0.01$) interaction between runs and cellulose digestion, acetate production and propionate production (Table 9). The variation between runs in Trial 3 in the magnitude of the response obtained upon

TABLE 7

Effects of deletion of PABA from the in vitro fermentation medium on cellulose digestion and VFA production

	Cellulose digested mg	VFA concentration (mmoles/100 ml) Acetate	Propionate	Butyrate
Complete medium	185	1.97	1.43	0.22
PABA deleted	174	2.02	1.25	0.20
Mean Squares:				
Treatment (1 df)	390**	0.0070	0.0990**	0.0012*
Error (10 df)	14.9	0.0149	0.00267	0.00018

*significant at $P < 0.05$
**significant at $P < 0.01$

TABLE 8

Effects of deletion of biotin from the fermentation medium on cellulose digestion and VFA production

	Cellulose digested mg	VFA concentration (mmoles/100 ml) Acetate	Propionate	Butyrate
Complete Medium	159	1.71	1.48	0.19
Biotin Deleted	45.7	1.15	0.48	0.17

TABLE 9

Mean squares for data of Table 8

Source	df	Cellulose[1] digestion	Acetate	Propionate	Butyrate
Treatment	1	191,500**	4.654**	14.93**	0.0052
Run	9	1,205**	0.4062**	0.02912**	0.00189
T x R	9	633.5**	0.06999**	0.06799**	0.00181
Error	40	52.84	0.01613	0.005763	0.00158

[1]one value lost
**significant at $P < 0.01$

deletion of biotin as well as the treatment by run interaction on cellulose digestion and acetate and propionate production are probably attributable to differences between runs in the amounts of biotin added with the inoculum and in the amount of biotin synthesized during the fermentation.

Discussion

These results are in agreement with those of Bentley et al. (1955) and Dehority et al. (1960) who obtained increased cellulose digestion by rumen microorganisms when PABA was added to a fermentation medium containing valeric acid. The reason for the decrease in propionate but not in acetate production upon deletion of PABA is not clear; PABA has not been specifically linked to any of the conversions which could give rise to propionate or propionate precursors.

Dehority et al. (1960) demonstrated that their washed preparations of rumen microorganisms required biotin for maximal cellulose digestion.

The removal of biotin from the fermentation medium in the present study gave rise to effects which were qualitatively and quantitatively very similar to those observed upon addition of 12 units of avidin to the fermentation tubes in Trial 2.

Summary

1. Deletion of PABA from the medium resulted in slightly decreased cellulose digestion, and propionate and butyrate production; acetate production was not influenced.

2. Deletion of biotin from the in vitro fermentation medium resulted in greatly decreased cellulose digestion, acetate production and propionate production. Propionate production was decreased to twice the extent that acetate production was decreased.

Discussion of Experiment I

A consideration of the possible biochemical routes of VFA production suggests the existence of a carbon flow system proceeding through glycolysis, with diversification occurring at some point in the glycolytic scheme with the lactate and the succinate pathways leading to propionate and a third pathway leading through pyruvate to acetate, and from acetate presumably to butyrate. Thiamine has been linked with the conversion of pyruvate to acetate. Biotin has been shown to be involved in a direct carboxylation of pyruvate to oxaloacetate, a transcarboxylation reaction resulting in the conversion of methylmalonyl-CoA and pyruvate to propionyl-CoA and oxaloacetate and a reversible carboxylation involving propionyl-CoA and methylmalonyl-CoA. All of these reactions for which biotin is a coenzyme could be fitted into a pathway leading from a glycolytic intermediate to propionate. Thus, upon the basis of potential routes of formation, it could be postulated that if one could block the function of biotin in the metabolism of the rumen organisms, the production of propionic acid would be inhibited. On the other hand, if one were to block the action of thiamine in this system, acetate and butyrate production should be inhibited. Experiment I was designed to test this hypothesis. It was found that the vitamin analogues oxythiamine, pyrithiamine, and desthiobiotin did not produce any effect on cellulose digestion or VFA production when present at very nearly substrate concentrations in the fermentation medium. If the problem was that the concentrations used were not great enough to show inhibitory effects, it is considered that the artificial rumen method is unsatisfactory for the study of the effects of these vitamin analogues upon mixed microbial metabolism. It is quite possible that the vitamin analogues were not effective because there was a mixed microbial population present; a given analogue could inhibit the

vitamin function in some organisms, but organisms not influenced by the analogue could flourish and thus mask any inhibitory effect.

Thiopental and hexetidine were effective inhibitors, hexetidine being capable of a complete inhibition of metabolism. It appeared that under some circumstances hexetidine inhibited acetate production, but not propionate production. This would be expected if thiamine were inhibited by this compound.

The addition of avidin to the medium and the removal of biotin from the medium resulted in similar effects, including a large reduction in cellulose digestion accompanied by a greater inhibition of propionate production than of acetate production; on the basis of these effects on fatty acid production, a biotin block could have occurred in a propionate-producing pathway. However, the results are confounded by influences upon cellulose digestion. It is not known if, or why, a block in the succinate route of propionate production would inhibit cellulose digestion. If, during cellulose digestion acetate and propionate were produced at different rates, relative to each other, inhibition of cellulose digestion might appear to affect the production of one acid to a greater extent than the other. Thus it is possible that biotin inhibition or biotin deletion resulted primarily in a block in cellulose digestion, rather than in a blockage of a propionate-producing pathway. This is a particularly pertinent consideration in view of the fact that removal of PABA from the medium resulted in a decrease in cellulose digestion and propionate production, but did not affect acetate production; PABA, unlike biotin, is not known to be directly associated with any reaction that could be involved in propionate production.

Experiment II

Rate of Cellulose Digestion and the Relationship of Acetate, Propionate and Butyrate Production to Cellulose Digested

Introduction and Object

It was demonstrated in Experiment I that the addition of avidin to or deletion of biotin from the medium resulted in a considerable reduction in cellulose digestion and VFA production by rumen microorganisms. There was a greater inhibition of propionate than of acetate production. It was suggested that these effects could have been the result of an inhibition of the action of biotin in a propionate pathway. However, the effects could have arisen from a non-specific inhibition of cellulose digestion if acetate and propionate were produced at different rates during cellulose digestion. Therefore, an experiment was carried out to study rates of cellulose digestion by rumen organisms *in vitro* and to determine the relationship between VFA production and cellulose digestion.

Experimental

Using a washed inoculum a run was carried out wherein all tubes contained the complete medium. Six tubes were removed from the water bath at each of 12, 18, 24 and 30 hours after initiation of fermentation. Upon removal, the tubes were treated as previously described (see general Experimental) to the point where the supernatant was discarded leaving only the insoluble cake in the tube. The tubes containing these cakes were stored at -15 C until all the tubes of the run had been taken off and cellulose determinations could be carried out on all the tubes at the same time.

Results

The relationship between hours after initiation of fermentation and mg of cellulose digested is illustrated in Fig. 5. The greatest portion of cellulose digestion occurred in the interval between 12 and 24 hours

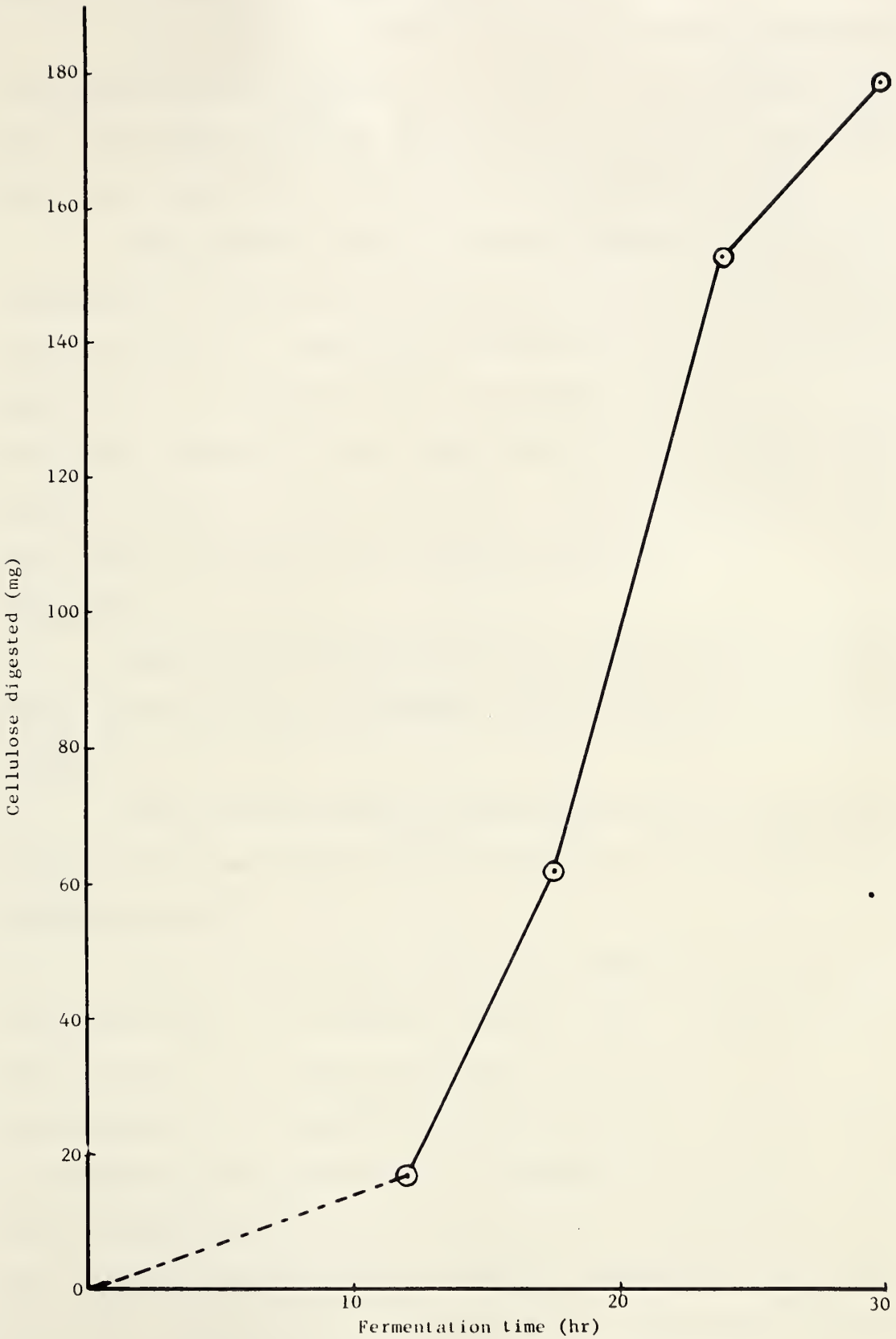

Figure 5. Rate of *in vitro* cellulose digestion.

after the initiation of fermentation.

The correlation coefficient, in the present run, between acetate concentration in the supernatant and weight of cellulose digested was 0.95, while that between propionate concentration and cellulose digested was 0.98. The corresponding coefficient for butyrate was only 0.24.

The regression curves for acetate, propionate and butyrate concentrations in the fermentation supernatant on amount of cellulose digested are shown in Fig. 6. Acetate and propionate were produced during cellulose digestion at rates which were not statistically different at P 0.05, using the t-test, although the rate of propionate production appeared slightly greater than that of acetate production. Butyrate concentration was only very slightly increased during cellulose digestion.

Discussion

The curve of cellulose digestion is the typical sigmoid bacterial growth curve and is in good agreement with the findings of Donefer et al. (1960).

The high correlation of acetate and propionate concentrations and weight of cellulose digested reflects production of these acids from the cellulose digested.

It is of significance that from extrapolation of the regression curves all three of the acids were present at zero cellulose digestion. This is only a very slight extrapolation, as the range of cellulose digestion covered in the experiment included 7 to 180 mg. The intercepts at 0.90 mmoles per 100 ml for acetate, 0.24 mmoles per 100 ml for propionate and 0.14 mmoles per 100 ml for butyrate at zero cellulose digestion probably represent the VFA production from the fermentation of the glucose of the medium, and conversion of some amino acids to VFA prior to the initiation of cellulose digestion.

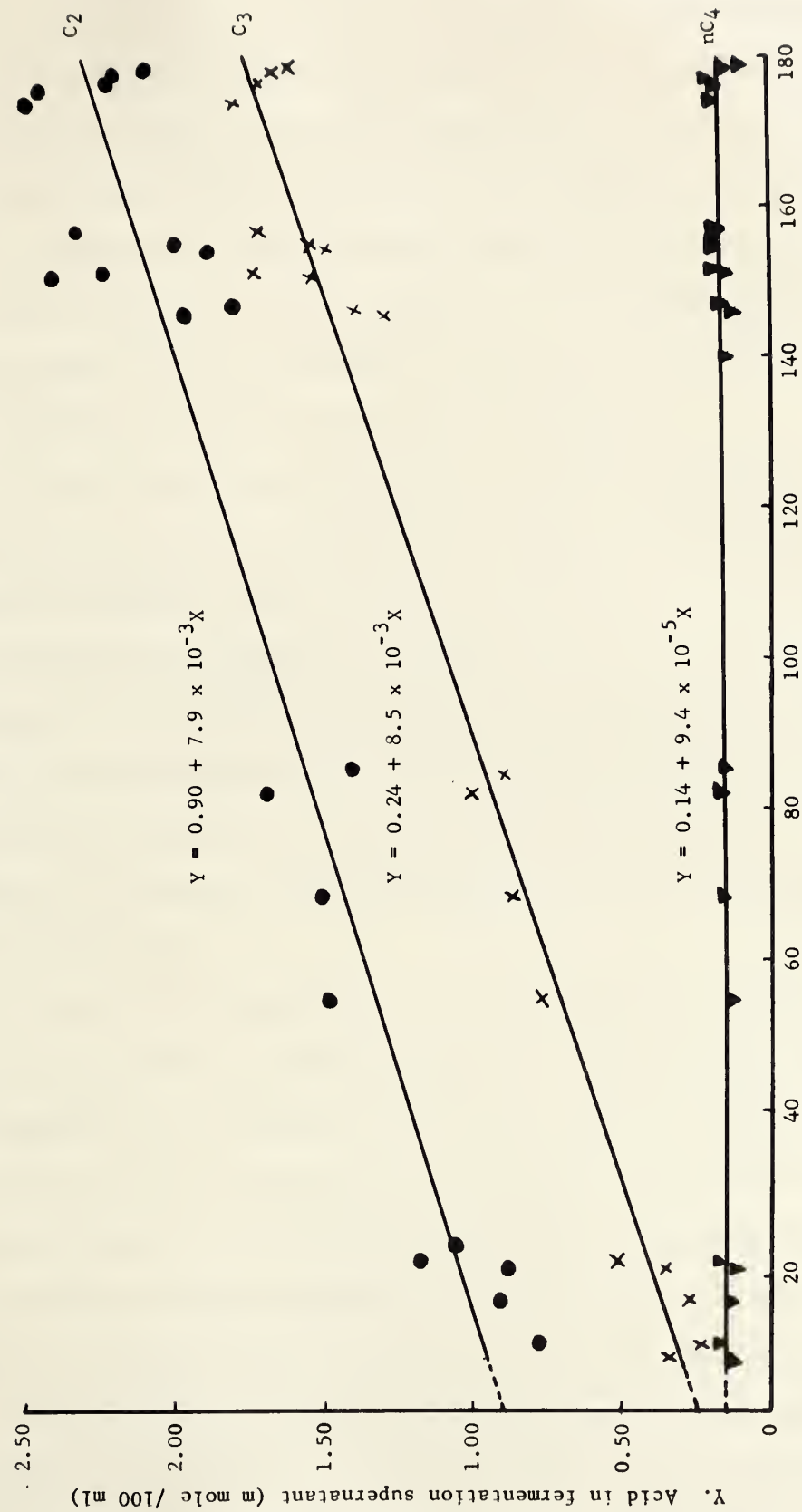

Figure 6. VFA concentration in the in vitro fermentation supernatant as a function of cellulose digested (C_2 = acetate; C_3 = propionate; nC_4 = n-butyrate)

The fact that butyrate concentration was influenced so slightly by cellulose digestion is difficult to reconcile with the assumption that butyrate is produced from the condensation of two molecules of acetate. If this were the case, it would be expected that the butyrate concentration would increase with increasing acetate production. Butyrate concentration increased only slightly while acetate concentration increased greatly with increased cellulose digestion. It is possible that in an isolated system there is a maximum to which butyrate production can proceed, regardless of the amount of precursor available.

If, in every run of Experiment I, acetate and propionate were produced at the same rate during cellulose digestion, as they were in the present experiment, then in those runs in which the final concentration of propionate was greater than that of acetate, there would have been a predominance of propionate produced during the 'pre-cellulose digestion' period. This would mean that the proportions of acid produced from the initial glucose fermentation must have varied greatly between runs in Experiment I because the final concentration of acetate and propionate varied considerably.

In 8 runs of Experiment I wherein biotin was inhibited, or deleted, and the VFA vs. cellulose digested regression lines calculated as in Appendix B, the average zero cellulose digestion VFA concentrations were 0.81 $\pm$ 0.24 mmoles acetate per 100 ml, 0.18 $\pm$ 0.05 mmoles of propionate and 0.14 $\pm$ 0.04 mmoles of butyrate. The final VFA concentrations of the complete medium control tubes for the 8 runs of Experiment I presently under discussion are given in Appendix A, Tables 1 to 8. It is evident from the zero cellulose digestion VFA concentrations that there was not sufficient variation between runs in the production of the VFA during the fermentation prior to cellulose digestion to result in

a greater concentration of propionate than acetate as was observed in the control tubes of 3 of the 8 runs. This suggests that in Experiment I, the rates of acetate and propionate production during cellulose digestion, differed relative to each other, between runs.

Indirect evidence from Experiment I did indicate that acetate could be produced at a different rate than propionate. When the rates of acetate and propionate production were calculated as in Appendix B from the data of the 8 runs above, in which biotin was inhibited, or deleted from the medium, it was found that acetate was produced at a rate of $5.7 \pm 0.6 \times 10^{-3}$ mmoles/100 ml per mg of cellulose digested, while propionate was produced at a rate of $8.7 \pm 0.7 \times 10^{-3}$ mmoles/100 ml per mg of cellulose digested. The average rate of acetate production in these runs was somewhat less than that observed in the present experiment, while the average rate of propionate production was essentially the same. This suggests that the rate of acetate production in the present experiment may have been unusually high. The greater rate of production of propionate as compared to acetate may be real in Experiment II as it was in Experiment I.

In general, if there was a greater production of propionate than of acetate per unit of cellulose digested, a reduction of cellulose digestion would reduce the units of propionate produced to a greater extent than those of acetate. On the other hand, if the production of acetate and of propionate was the same per unit of cellulose digested, as suggested in the present experiment, but the 'pre-cellulose digestion' production rates varied, then it would have to be concluded that biotin inhibition or deletion in Experiment I gave rise to a metabolic block in the production of propionate.

Summary

1. The major part of cellulose digestion occurred in the interval from 12 to 24 hours after the initiation of *in vitro* fermentation with washed rumen microorganisms.

2. Acetate production and propionate production were highly correlated with the weight of cellulose digested. Butyrate production was only very slightly influenced by cellulose digestion.

3. Acetate was the predominant acid produced in fermentation occurring prior to cellulose digestion.

4. Propionate was not produced at a significantly greater rate than acetate during cellulose digestion.

5. Data from Experiment I suggested that propionate was usually produced at a greater rate than acetate during *in vitro* cellulose digestion by rumen microorganisms.

Experiment III

Effects of Some Metabolic Intermediates on Cellulose Digestion in the Presence of Added Biotin and in Biotin Deficiency and the Conversion of the Intermediates to VFA in the Presence of Added Biotin and in Biotin Deficiency.

Introduction and Object

In Experiment I it was found that the addition of avidin to a complete fermentation medium, or the deletion of biotin from the fermentation medium resulted in the inhibition of cellulose digestion by rumen microorganisms. It was also found that VFA production was inhibited; propionate production being inhibited to a greater extent than acetate production.

It has been shown (Kaziro et al., 1960; Swick and Wood, 1960; Bloom and Johnson, 1962) that biotin is a cofactor for several enzymes which could be involved in the succinate route of propionate formation.

Experiment III was designed in an attempt to provide evidence regarding the functional routes of VFA production by studying the conversion to VFA of some of the intermediates of proposed VFA-producing pathways. It was also thought that knowledge of the effects of the intermediates upon cellulose digestion in a biotin deficient system and of the effects of biotin deficiency upon the conversion of the intermediates to VFA might provide evidence for the existence of a biotin-dependent conversion in this system. This approach should also demonstrate if biotin deficiency inhibited cellulose digestion and thereby inhibited VFA production or if biotin deficiency inhibited VFA production and thereby inhibited cellulose digestion.

Experimental

Distilled water was substituted for the regular biotin solution in the media for all the runs of Experiment III, excluding run 15. In run 15, the regular biotin-containing medium was used.

The runs of Experiment III were designed basically the same as described in the general Experimental, with 8 treatments, including controls, at 3 tubes per treatment comprising a run. An intermediate was always studied in the presence of added biotin and in a biotin deficient treatment within any one run. In each run there were control tubes with biotin added, which will be referred to as complete medium controls, and biotin deficient controls. Each intermediate was studied in at least 2 separate runs.

The metabolic intermediates used in Experiment III were: pyruvic acid[1], lactic acid[1], L-malic acid[1], fumaric acid[1], succinic acid[1], oxaloacetic acid[2], glucose[1], and 3-phosphoglycerate[2,3].

Glucose was used at a level of 0.28 mmoles per fermentation tube; the rest of the intermediates were used at a level of 0.55 mmoles per fermentation tube. Presumably 0.28 mmoles of glucose could be degraded to 0.56 mmoles of triose.

The readily soluble intermediates including glucose, pyruvic acid, lactic acid, oxaloacetic acid, malic acid and succinic acid were added in aqueous solution to the fermentation tubes immediately prior to dispensing the medium and inoculum. Four ml of solution, containing the required amount of a precursor plus 1 ml of either distilled water or a solution containing 10 ug of biotin were added to each treatment tube. To the complete medium control tubes were added 4 ml of distilled water plus 1 ml of biotin solution, while 5 ml of distilled water only were added

[1]Fisher Scientific Co. Ltd., 14730 - 115A Ave., Edmonton, Alta.
[2]California Corporation for Biochemical Research, 3625 Medford Street, Los Angeles 63, Calif.
[3]purchased as 3-phosphoglycerate (Barium salt) $\cdot 2H_2O$

to the biotin deficient control tubes.

In run 15 addition of 12 units of avidin rather than biotin deletion was used to produce the biotin deficiency. In this run, 4 ml of precursor solution plus 4 ml of either distilled water or a 3 unit per ml solution of avidin were added to each treatment tube. To the complete medium control tubes were added 8 ml of distilled water while the deficient control tubes had 4 ml of distilled water plus 4 ml of avidin solution added.

Fumaric acid was found to be rather insoluble. Therefore 0.55 mmoles of this compound (63.7 mg) were weighed directly into the fermentation tubes prior to fermentation, and 4 ml of distilled water were added.

The sodium salt of 3-phosphoglycerate was prepared as follows: 13.75 mmoles of 3-phosphoglycerate $\cdot 2H_2O$, barium salt, were weighed into a 50 ml volumetric flask. To this was added approximately 30 ml of distilled water. Dilute HCl was then added very slowly, with shaking, in a sufficient quantity to bring the white salt into solution. Ten ml, containing 7.0 mmoles, of a solution of Na_2SO_4 were added to the volumetric flask and the flask was filled to volume with distilled water. The $BaSO_4$ was allowed to precititate for 18 hr at 2 C, after which time the solution was filtered repeatedly through Whatman no. 42 filter paper. The clear filtrate solution, 4 ml of which contained 0.55 mmoles of 3-phosphoglycerate as the sodium salt, was used as the additive for the tubes in which the metabolism of 3-phosphoglycerate was to be studied. Washed inocula were used for all the runs of Experiment III.

The results of the conversion of precursors to VFA, used in the report of this experiment have all been adjusted for differences from the controls in cellulose digestion and converted to a fraction of the total precursor recovery by the method described in Appendix B. The values for

any precursor will therefore represent the recovery of 1.00 units of VFA, from the metabolism of the precursor, the proportions of individual acids being the same as were produced from that precursor.

Results

All the raw results of Experiment III are presented in Appendix A, Tables 1 to 8.

a. Cellulose Digestion

The effects of the VFA precursors on cellulose digestion in the presence and absence of biotin are summarized in Table 10. As is reflected by the large standard errors of the values in Table 10, there was considerable variation between runs in the response to any particular intermediate. The number of runs in which an intermediate was used can be determined by adding one to the number of degrees of freedom given for that intermediate.

In the presence of biotin, oxaloacetic acid at a concentration of 0.011 M in the fermentation mixture, decreased cellulose digestion by approximately 35 per cent, while 0.011 M 3-phosphoglycerate decreased cellulose digestion by approximately 20 per cent. When biotin was added to the medium, 0.0056 M glucose, 0.011 M pyruvic, lactic, malic, fumaric or succinic acid had slight effects upon cellulose digestion; the direction of the response of cellulose digestion to these intermediates was inconsistent between runs.

In a biotin deficient system, pyruvic acid caused a stimulation of cellulose digestion of the order of 60 per cent, while malic acid stimulated cellulose digestion by approximately 40 per cent. Lactic acid and 3-phosphoglycerate appeared to stimulate cellulose digestion slightly in the biotin deficient tubes. Glucose, oxaloacetic acid, fumaric acid, or succinic acid resulted in rather small effects, the directions of which were unpredictable.

TABLE 10

Effects of VFA precursors on cellulose digestion in the presence and absence of biotin

Precursor	df	Biotin added avg difference from control mg of cellulose	SE	Biotin deficient avg difference from control mg of cellulose	SE
Glucose	2	-2.4	3.8	-1.6	3.3
3-Phosphoglycerate	1	-28.3*	11.0	7.5*	2.5
Pyruvic acid	2	5.0	3.8	28.3***	8.2
Lactic acid	1	3.9	8.7	12.5**	5.1
Oxaloacetic acid	1	-51.5***	2.9	-5.0	6.6
Malic acid	2	-5.3	9.9	16.3***	4.9
Fumaric acid	1	-12.3	7.8	-3.2	12.4
Succinic acid	1	2.6	5.4	5.6	5.6

*t significant at $P < 0.15$
**t significant at $P < 0.10$
***t significant at $P < 0.05$

TABLE 11

Conversion of glucose to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units/units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 18	complete	0.26	0.25	0.49	0.77
	biotin deleted	0.17	0.20	0.63	1.01
Run 19	complete	-0.02	0.56	0.45	0.62
	biotin deleted	0.24	0.44	0.32	0.87
Run 21	complete	0.19	0.43	0.38	0.74
	biotin deleted	0.14	0.37	0.48	0.62

[1]total VFA recovered from the metabolism of the glucose

b. Conversions to VFA

It was assumed that an increase as compared to the control tube, in the concentration of any of the VFA in a tube to which had been added a metabolic intermediate, represented the conversion of the intermediate to that acid. It is possible that the intermediate stimulated the more efficient production of VFA from cellulose digestion, and therefore the carbon recovered as fatty acids may not be the carbon added as the intermediate. The validity of this argument can not be judged at this time.

Glucose. In the presence of added biotin, glucose was converted to acetate, propionate and butyrate (Table 11). Biotin deletion resulted in a decreased recovery of propionate from glucose. In runs 18 and 21, deletion of biotin decreased the proportion of acetate and increased the proportion of butyrate in the VFA recovered from glucose metabolism. In run 19, biotin deletion increased the proportion of acetate and decreased the proportion of butyrate.

3-Phosphoglycerate. Acetic acid was the predominant product of 3-phosphoglycerate metabolism (Table 12). There was no conversion of 3-phosphoglycerate to propionate in the presence of added biotin. This conversion was observed upon deletion of biotin. The proportion of VFA recovered as butyrate was less in the presence than in the absence of added biotin, while the proportion recovered as acetate was decreased upon deletion of biotin.

Pyruvic acid. As for 3-phosphoglycerate, acetate was the predominant acid produced from pyruvic acid metabolism (Table 13). In runs 15 and 18 biotin deficiency decreased the proportion of propionate, and increased the proportion of butyrate. In run 19, biotin removal increased the proportion of propionate and decreased the proportion of butyrate. The effect of biotin upon acetate production from pyruvic acid was completely random.

TABLE 12

Conversion of 3-phosphoglycerate to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 20	complete	1.22	-0.34	0.13	0.32
	biotin deleted	0.57	0.11	0.31	0.70
Run 24	complete	1.26	-0.26	0	0.50
	biotin deleted	0.82	0.02	0.16	0.89

[1]total VFA recovered from the metabolism of the 3-phosphoglycerate

TABLE 13

Conversion of pyruvic acid to VFA in the presence of added biotin and in biotin deficiency

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 15	complete	0.78	0.14	0.08	0.96
	12 units avidin	0.97	-0.09	0.12	0.66
Run 18	complete	0.83	0.05	0.12	0.81
	biotin deleted	0.83	-0.03	0.20	0.69
Run 19	complete	0.87	-0.20	0.33	0.55
	biotin deleted	0.71	0.02	0.27	0.90

[1]total VFA recovered from the metabolism of the pyruvic acid

Lactic acid. Butyrate accounted for a large portion of the VFA formed from the fermentation of lactic acid (Table 14). The conversion of lactic acid to butyrate was greater than the conversion of lactic acid to propionate. Biotin removal decreased the conversion of lactic acid to butyrate in runs 20 and 26, but increased this conversion in run 21. Lactic acid was converted to acetate and propionate, but the proportion of these acids produced was variable, and the influence of biotin upon these conversions was inconsistent.

Oxaloacetic acid. The acetate produced upon the fermentation of oxaloacetic acid accounted for very nearly 100 per cent of the oxaloacetic acid recovered as VFA (Table 15). Deletion of biotin from the fermentation medium resulted in an increase in the proportion of propionate and butyrate produced from oxaloacetic acid. When biotin was not added to the fermentation mixture, about 70 to 80 per cent of the VFA produced from oxaloacetate fermentation was acetate.

Malic acid. Propionate consistently accounted for 60 to 70 per cent of the VFA produced from malic acid when this compound was fermented in the presence of biotin (Table 16). Acetate was a significant product of malate fermentation while only a small proportion of butyrate was produced. In runs 20 and 21 the deletion of biotin from the fermentation system resulted in an increase in the proportions of acetate and butyrate and a decrease in the proportion of propionate produced from malate. Conversely in runs 23 and 26, upon deletion of biotin, there was an increase in the proportion of propionate and a decrease in the proportion of acetate and butyrate produced from malate.

Fumaric acid. In the presence of biotin the predominant acid produced from the fermentation of fumaric acid was propionate (Table 17). Acetate was also produced upon fumarate fermentation, and, in the presence of biotin,

TABLE 14

Conversion of lactic acid to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 20	complete	-0.29	0.24	1.06	0.34
	biotin deleted	0.23	0.17	0.60	0.60
Run 21	complete	0.13	0.29	0.58	0.52
	biotin deleted	0.12	0.22	0.65	0.49
Run 26	complete	0.33	0.15	0.51	0.78
	biotin deleted	0.28	0.27	0.46	1.01

[1]total VFA recovery from the metabolism of the lactic acid

TABLE 15

Conversion of oxaloacetic acid to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 23	complete	0.97	0.01	0.02	0.86
	biotin deleted	0.88	0.07	0.05	0.85
Run 24	complete	1.09	-0.09	0	0.57
	biotin deleted	0.78	0.09	0.13	1.05

[1]total VFA recovery from the metabolism of the oxaloacetic acid

TABLE 16

Conversion of malic acid to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 20	complete	0.21	0.66	0.14	0.58
	biotin deleted	0.33	0.44	0.22	1.08
Run 21	complete	0.21	0.71	0.08	0.52
	biotin deleted	0.36	0.49	0.14	0.85
Run 23	complete	0.38	0.56	0.07	1.17
	biotin deleted	0.23	0.80	-0.03	0.70
Run 26	complete	0.35	0.59	0.06	1.08
	biotin deleted	0.22	0.76	0.02	0.94

[1]total VFA recovery from the metabolism of the malic acid

TABLE 17

Conversion of fumaric acid to VFA in the presence and absence of added biotin

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 23	complete	0.42	0.54	0.07	0.92
	biotin deleted	0.70	0.48	-0.19	0.54
Run 24	complete	0.19	1.13	-0.32	0.31
	biotin deleted	0.51	0.56	0.08	1.05

[1]total VFA recovery from the metabolism of the fumaric acid

TABLE 18

Conversion of succinic acid to VFA in the presence of added biotin and in biotin deficiency

	Treatment	Proportional fatty acid recoveries (units / units TVFA) Acetate	Propionate	Butyrate	TVFA[1] recovery acid units/100 ml
Run 15	complete	-0.10	1.10	0	0.82
	12 units avidin	0	0.96	0.04	0.99
Run 18	complete	-0.11	1.13	-0.03	0.74
	biotin deleted	0.07	0.85	0.08	0.99

[1]total VFA recovered from the metabolism of the succinic acid

the fermentation of fumaric acid inhibited the production of butyrate. The deletion of biotin from the fermentation resulted in a decreased conversion of fumaric acid to propionate and an increased conversion to acetate. In run 23, biotin deletion decreased the proportion of butyrate produced from fumaric acid, but in run 24 the proportion of butyrate was increased upon deletion of biotin.

Succinic acid. The fermentation of succinic acid in the presence of biotin resulted in the formation of propionate only (Table 18). There appeared to be an inhibition of acetate formation when succinic acid was present and there was no conversion of succinic acid to butyrate. In the absence of biotin there was a decrease in the conversion of succinic acid to propionate and slight conversion of succinic acid to acetate and butyrate.

Discussion

Hershberger et al. (1956) reported that at a concentration of 0.02 M in the fermentation mixture, glucose, pyruvate, succinate, or malate did not influence cellulose digestion by bovine rumen microorganisms. These results were substantiated in the present study, when it was demonstrated that glucose (0.0056 M in the fermentation mixture), pyruvic acid (0.011 M), succinic acid (0.011 M), or malic acid (0.011 M) resulted in only small, inconsistent, effects on cellulose digestion.

Hershberger et al. (1956) also found that lactic acid was very inhibitory to cellulose digestion. In the present experiment lactic acid did not inhibit cellulose digestion. However, the concentration of lactic acid used by Hershberger et al. (1956) was twice that used in the present experiment.

The very marked depression of cellulose digestion by oxaloacetic acid is not readily explainable, especially when the other tricarboxylic

acid cycle intermediates, malic, fumaric and succinic acids, did not depress cellulose digestion. Payes and Laties (1963) found that oxaloacetic acid and glyoxylate condense non-enzymatically to form γ-hydroxy- α-ketoglutaric acid, a powerful inhibitor of aconitase, isocitric dehydrogenase and α-ketoglutaric dehydrogenase. Oxaloacetic acid may have condensed with glyoxylate in the present study but it is not known what significance the γ-hydroxy- α-ketoglutarate-sensitive enzymes may have in this anaerobic fermentation.

The observed inhibition of cellulose digestion in the presence of 3-phosphoglycerate may have been attributable to incomplete removal of the barium from the original barium salt. Also, in addition to 3-phosphoglycerate, fairly high levels of Na^+ and Cl^- were added to these fermentation tubes because HCl was used to get the barium salt into solution, and Na_2SO_4 was used to precipitate the barium, leaving the Cl^- and Na^+ in solution with the 3-phosphoglycerate. It was not expected that these ions would influence cellulose digestion, but they do contribute a confounding factor.

It was hypothesized that, in a system in which biotin-dependent reactions were blocked, the addition of intermediates on the cellulose side of the block would not stimulate cellulose digestion, while the addition of intermediates on the opposite side of the block might stimulate digestion. It was found that malic acid and pyruvic acid stimulated cellulose digestion in the biotin deficient system. Lactic acid and 3-phosphoglycerate were somewhat less effective. On the other hand, glucose, oxaloacetic acid, fumaric acid and succinic acid did not stimulate cellulose digestion in the biotin deficient system. These results do not indicate the existence of a specific biotin deficiency block in any known sequence of reactions or in the possible pathways of VFA formation.

The potential routes of VFA production have been pieced together using information from a great many studies, but it has yet to be established which routes are actually functional in the rumen metabolic scheme. It was considered that an insight into this problem could be gleaned by determining the conversion of some of the possible VFA precursors to the individual fatty acids. The finding that glucose was converted to acetate, propionate and butyrate by bovine rumen microorganisms is in agreement with the work of Doetsch et al. (1953) and Hershberger et al. (1956).

It was expected that either phosphoenolpyruvate or pyruvate would be the final glycolytic precursor of the succinic route of propionate formation. The finding that both 3-phosphoglycerate and pyruvic acid were converted almost exclusively to acetate does not agree with this hypothesis. The results obtained from the fermentation of 3-phosphoglycerate and pyruvic acid do not provide evidence to suggest that these compounds might be involved in a route leading to the production of succinate precursors in the artificial rumen. It is possible that the method used in these studies gives unrealistic conversions because the fermentations were initiated with a nonphysiological concentration of one intermediate. If the CO_2 fixation enzymes become saturated with substrate at a much lower substrate concentration than that required for the saturation of the acetate route, it might be expected that the major part of the excessive intermediate would be converted to acetate. This disproportionate conversion would be magnified if the rate constants of the systems differed, with that for the acetate route being greater than that for the propionate route. It might have been more revealing if the intermediates had been added over a period of time after the initiation of fermentation when the various substrate pools of the system had accumulated. Under these circumstances the addition of the one substrate would not be so likely to put such an unnatural

stress upon the system.

The very nearly quantitative conversion of oxaloacetic acid to acetate and the inhibition of cellulose digestion by oxaloacetic acid have not previously been reported. The observation on the conversion of oxaloacetic acid to acetate would cast doubt upon the significance of this intermediate as a propionate precursor. It would be implied that the sequence involving carboxylation of phosphoenolpyruvate or pyruvate to oxaloacetate and conversion of this to succinate is not operative as a route of propionate formation in the artificial rumen. At the same time it could be argued that the formation of acetate from oxaloacetic acid was an unnatural conversion precipitated by the stress of the high concentration of the intermediate, such as was suggested for pyruvic acid and 3-phosphoglycerate conversions. It was expected that pyruvic acid and 3-phosphoglycerate would be at least partially converted to acetate; that is, the acetate route was a known alternate for the conversion of these precursors. The conversion of oxaloacetic acid to acetate was not expected, and the manner in which this conversion could have occurred is rather obscure. Oxaloacetic acid may have been decarboxylated to pyruvate which in turn would have been converted to acetate, but this would not explain the inhibition of cellulose digestion caused by oxaloacetic acid. Pyruvic acid, it will be recalled, did not inhibit cellulose digestion.

The results obtained on the conversion of malic acid and fumaric acid differ somewhat from those reported by Doetsch et al. (1953) who found that these intermediates were converted primarily to acetate by rumen microorganisms. In the present experiment acetate was produced, but the major VFA product of the fermentation of malic acids was propionate. It would appear that malic and fumaric acid can be converted to succinate

and thence to propionate, or they can be converted to acetate perhaps by way of oxaloacetate or conversion to a glycolytic intermediate.

Succinic acid was converted to propionate only; this is in agreement with a number of other reports. It is interesting to note that the conversion of succinic acid to propionate was associated with a reduction in the amount of acetate produced. This eliminates any possibility of central cleavage of succinate to acetate in the present experiment.

A transcarboxylation reaction has been demonstrated which involves the simultaneous conversion of methylmalonyl-CoA and pyruvate to propionyl-CoA and oxaloacetate. This could be used to explain the predominant conversion of pyruvic acid and 3-phosphoglycerate to acetate, because, for these intermediates to have been converted to propionate, if transcarboxylation is involved in propionate formation, a source of methylmalonyl-CoA would have been necessary. Similarly, when succinic acid was the added substrate, its conversion to methylmalonyl-CoA, followed by transcarboxylation would direct pyruvate units away from acetate production and toward the formation of tricarboxylic acid cycle intermediates. The system would not imply a continuous cycle as it was shown that some of the tricarboxylic acid cycle intermediates are converted to acetate by way of a route which may not involve pyruvate. Conversion of the tricarboxylic acid cycle intermediates to acetate would also mean that the reduction in acetate production would not need to be equivalent to the amount of methylmalonyl-CoA-producing metabolite added. An isolated transcarboxylation system would require that the moles of pyruvate directed away from acetate production be equivalent to the exogenous methylmalonyl-CoA added.

It was observed that lactic acid was converted to acetate, propionate and butyrate. Similar results have been reported by other workers.

The most notable aspect of the lactic acid conversion data is the large proportion of butyrate in the VFA produced from lactic acid. The proportion of butyrate produced from lactic acid was greater than for any of the other intermediates studied, including those which were converted nearly quantitatively to acetate, although there was a sizeable proportion of butyrate produced from glucose. This suggests that there may be a relationship between lactic acid and butyrate which does not involve acetate as an intermediate, but this possibility certainly requires further clarification. The large conversion of lactic acid to butyrate has not been observed by other workers.

Biotin deficiency was found to produce very inconsistent effects upon the proportions of the individual fatty acids produced from lactic, pyruvic and malic acids. Biotin deficiency did appear to decrease the proportion of propionate produced from succinic acid, fumaric acid and glucose, but stimulated the production of propionate from 3-phosphoglycerate. These results can not be interpreted as a specific effect upon a propionate-producing system. It would appear that the experimental method used was incapable of showing any biotin deletion effects at a specific metabolic level. One weakness could have arisen from the fact that the entire quantity of each of the intermediates used was added prior to the initiation of fermentation. There was certainly endogenous biotin added with the inoculum and this endogenous biotin may have been sufficient for the metabolism of the readily available intermediates. A metabolic block due to a lack of biotin may have developed only during a later stage of fermentation when the endogenous biotin was depleted. Thus the studies on the effect of biotin on the conversions of the metabolic intermediates might have been more valuable if the intermediates had been added to the fermentation tubes at a later stage of fermentation.

Summary:

1. The presence of lactic acid, pyruvic acid, succinic acid, malic acid or fumaric acid at a level of 0.011 M, or glucose at a level of 0.0056 M in a complete fermentation medium resulted in only slight, inconsistent effects upon cellulose digestion by bovine rumen organisms. Oxaloacetic acid (0.011 M) and 3-phosphoglycerate (0.011 M) decreased cellulose digestion.

2. Pyruvic and malic acids stimulated cellulose digestion when present in a biotin deficient medium at a concentration of 0.011 M. In a biotin deficiency, it appeared that 3-phosphoglycerate and lactic acid would stimulate cellulose digestion very slightly. Other metabolic intermediates did not noticeably influence the cellulose digestion supported by a biotin deficient medium.

3. All of the intermediates were converted to VFA but the conversion of some, including 3-phosphoglycerate, pyruvic acid and oxaloacetic acid, was not in agreement with proposed pathways.

4. The effects of biotin deficiency on the conversion of the intermediates to VFA were rather inconsistent. A biotin deficiency block was not evident from the effect of biotin deletion or inhibition upon precursor conversions.

General Discussion

The fact that the treatments to which the mixed microbial populations were exposed would modify the relative numbers of the various types of organisms within a population has not been ignored.

An individual microorganism would not be capable of carrying out all the conversions that are known to take place in the rumen. Similarly, one enzyme or enzyme system is not capable of carrying out all the conversions known to occur within a cell. It has often been demonstrated that the presentation of a substrate to a growing cell can result in the induction of an enzyme or enzyme system. It has been suggested that presentation of a particular substrate to a rumen population causes one species or group of organisms to flourish or, in other words, causes an increased synthesis of the enzymes involved in the metabolism of that substrate. Thus the biochemical situation in the rumen is rather similar to that of the cell. It is therefore considered that interpretation of results of artificial rumen studies in a strictly biochemical light is justified, because, even though a substrate may cause an increase in an enzyme system (increased relative numbers of an organism) it would be metabolized, presumably, by routes which are potentially available under normal conditions.

The use of mixed rumen bacterial populations in metabolic studies offers unique advantages. It is evident from the present studies that the enzymes of the system are easily accessible to a number of substrates. This is an advantage in metabolic studies that is characteristic of cell-free extracts. The metabolism in the rumen is intimately concerned with growth, and therefore must be kept within fairly narrow limits of stress and in a delicate dynamic balance. This is a situation encountered in metabolic studies carried out with intact macroorganisms and it often makes

the interpretation of experimental results rather difficult, for there are usually a considerable number of alternative routes for the metabolism of any compound in a wholly functional system. The results obtained upon addition of the precursors to the fermentation tubes demonstrate that there are a number of alternate conversions in the metabolic scheme of the artificial rumen for many of the intermediates. A mixed culture of rumen bacteria presents a biochemical situation which approaches the complexity of the metabolic interrelationships found in an intact macroorganism but which is readily accessible for study under wholly functional conditions.

General Conclusions

The fact that competitive metabolic antagonists were not effective in this study, but avidin, a non-competitive antagonist was, would indicate that the concentrations of competitive antagonists used were not great enough to show any effects.

It is considered that future work could be profitably spent in determining the site of action of hexetidine in the metabolism in the artificial rumen.

A blockage of a specific conversion of a propionate-producing pathway in a biotin deficiency was not demonstrated, but this could not be taken as evidence that biotin is not involved with propionate formation in the artificial rumen. If the intermediates were added to the artificial rumen over a period of time during a later stage of fermentation, it is possible that a biotin deficiency could be shown to affect a specific conversion.

It would certainly be of interest and value to direct studies towards the clarification of the relationship between acetate and oxaloacetic acid and of that between butyrate and lactic acid.

The reason for the inhibition of cellulose digestion by oxaloacetic acid requires further investigation.

BIBLIOGRAPHY

Ables, P. G., J. M. Ravel, and W. Shive. 1961. The indirect role of biotin in the synthesis of the malic enzyme. J. Biol. Chem. 236: 3263.

Ajl, S. J., W. R. Hart, and C. H. Werkman. 1950. Biotin in succinic acid oxidation. Enzymologia 14: 1.

Allen, S. H. G., R. W. Kellermeyer, R. Stjernholm, and H. G. Wood. 1963. Partial reconstruction of the propionic acid fermentation with purified enzymes. Federation Proc. 22: 355.

Allen, S. H. G., H. G. Wood, R. Stjernholm, and R. W. Kellermeyer. 1962. Separation and purification of methylmalonyl Co A racemase and isomerase from propionic acid bacteria. Federation Proc. 21: 244.

Annison, E. F., and D. Lewis. 1959. Metabolism in the rumen. John Wiley and Sons, Inc., New York. 184 p.

Baldwin, R. L., W. A. Wood, and R. S. Emery. 1962. Conversion of lactate-C^{14} to propionate by the rumen microflora. J. Bacteriol. 83: 907.

Baldwin, R. L., W. A. Wood, and R. S. Emery. 1963. Conversion of glucose-C^{14} to propionate by the rumen microbiota. J. Bacteriol. 85: 346.

Bandurski, R. S., and C. M. Greiner. 1953. The enzymatic synthesis of oxalacetate from phosphoryl-enolpyruvate and carbon dioxide. J. Biol. Chem. 204: 781.

Barker, H. A., and F. Lipmann. 1944. On lactic acid metabolism in propionic acid bacteria and the problem of oxido-reduction in the system fatty-hydroxy-keto acid. Arch. Biochem. 4: 361.

Barnett, A. J. G., and R. L. Reid. 1961. Reactions in the rumen. Edward Arnold (Publishers) Ltd. London. 252 p.

Bentley, O. G., R. R. Johnson, T. V. Hershberger, J. H. Cline, and A. L. Moxon. 1955. Cellulolytic-factor activity of certain short-chain fatty acids for rumen microorganisms in vitro. J. Nutrition 57: 389.

Blackburn, T. H., and R. E. Hungate. 1963. Succinic acid turnover and propionate production in the bovine rumen. Appl. Microbiol. 11: 132.

Blanchard, M. L., S. Korkes, A. del Campillo, and S. Ochoa. 1950. Function of biotin in the metabolism of Lactobacillus arabinosus. J. Biol. Chem. 187: 875.

Bloom, S. J., and M. J. Johnson. 1962. The pyruvate carboxylase of Aspergillus niger. J. Biol. Chem. 237: 2718.

Bruno, C. F., and W. E. C. Moore. 1962. Fate of lactic acid in rumen ingesta. J. Dairy Sci. 45: 109.

Cantarow, A., and B. Schepartz. 1962. Biochemistry. 3rd. ed. W. B. Saunders Co., Philadelphia, Penn. 938 p.

Cerecedo, L. R. 1955. Thiamine antagonists. Amer. J. Clin. Nutrition 3: 273.

Crampton, E. W., and L. A. Maynard. 1938. The relation of cellulose and lignin content to the nutritive value of animal feeds. J. Nutrition 15: 383.

Dehority, B. A., K. el-Shazly, and R. R. Johnson. 1960. Studies with the cellulolytic fraction of rumen bacteria obtained by differential centrifugation. J. Animal Sci. 19: 1098.

DeLey, J., and J. Schell. 1962. Lactate and pyruvate catabolism in acetic acid bacteria. J. Gen. Microbiol. 29: 589.

Delwiche, E. A. 1948. Mechanism of propionic acid formation by Propionibacterium pentosaceum. J. Bacteriol. 56: 811.

Delwiche, E. A., E. F. Phares, and S. F. Carson. 1953. Succinate decarboxylation reaction in propionibacterium. Federation Proc. 12: 194.

Delwiche, E. A., E. F. Phares, and S. F. Carson. 1954. Succinate decarboxylation systems in propionibacterium and veillonella. Federation Proc. 13: 198.

Doetsch, R. N., R. Q. Robinson, R. E. Brown, and J. C. Shaw. 1953. Catabolic reactions of mixed suspensions of bovine rumen bacteria. J. Dairy Sci. 36: 825.

Donefer, E., E. W. Crampton, and L. E. Lloyd. 1960. Prediction of the nutritive value index of a forage from in vitro rumen fermentation data. J. Animal Sci. 19: 545.

Elsden, S. R., and D. Lewis. 1953. The production of fatty acids by a gram-negative coccus. Biochem. J. 55: 183.

Elsden, S. R., and A. K. Sijpesteijn. 1950. The decarboxylation of succinic acid by washed suspensions of rumen bacteria. J. Gen. Microbiol. 4: xi.

Elsden, S. R., B. E. Volcani, F. M. G. Gilchrist, and D. Lewis. 1956. Properties of a fatty acid forming organism isolated from the rumen of sheep. J. Bacteriol. 72: 681.

Fahmy, A. R., H. H. Ibrahim, and M. Talaat. 1962. Inhibitory effect of thiopentone on the pyruvic dehydrogenase in brain. Reversibility of action by thiamine pyrophosphate and lipoic acid. Can. J. Biochem. Physiol. 40: 477.

Flavin, M., and S. Ochoa. 1957. Metabolism of propionic acid in animal tissues. I. Enzymatic conversion of propionate to succinate. J. Biol. Chem. 229: 965.

Halenz, D. R., J. Y. Feng, C. S. Hegre, and M. D. Lane. 1962. Some enzymic properties of mitochondrial propionyl carboxylase. J. Biol. Chem. 237: 2140.

Halliwell, G. 1961. Carbohydrate metabolism in the rumen, p. 119-126. In D. Lewis (ed.), Digestive physiology and nutrition of the ruminant. Butterworth and Co. (Publisher) Ltd., London.

Halvorson, H. H., and B. D. Church. 1957. Intermediate metabolism of aerobic spores. II. The relationship between oxidative metabolism and germination. J. Appl. Bacteriol. 20: 359.

Hershberger, T. V., O. G. Bentley, J. H. Cline, and W. J. Tyznik. 1956. Formation of short-chain fatty acids from cellulose, starch, and metabolic intermediates by ovine and bovine rumen microorganisms. J. Agr. Food Chem. 4: 952.

Holzer, H., F. da Fonseca-Wollheim, G. Kohlhaw, and Ch. W. Woenckhaus. 1962. Active forms of acetaldehyde, pyruvate and glycolic aldehyde. Ann. N. Y. Acad. Sci. 98: 453.

Howard, B. H. 1959. Metabolism of carbohydrates by rumen bacteria. Proc. Nutrition Soc. 18: 103.

Hueter, F. G., R. J. Gibbons, J. C. Shaw, and R. N. Doetsch. 1958. Comparison of *in vivo* and *in vitro* techniques in rumenology studies. J. Dairy Sci. 41: 651.

Hueter, F. G., J. C. Shaw, and R. N. Doetsch. 1956. Absorption and dissimilation of lactates added to the bovine rumen and the resulting effects on blood glucose. J. Dairy Sci. 39: 1430.

Jayasuriya, G. C. N., and R. E. Hungate. 1959. Lactate conversions in the bovine rumen. Arch. Biochem. and Biophys. 82: 274.

Johns, A. T. 1951 a. The mechanism of propionic acid formation by *Veillonella gazogenes*. J. Gen. Microbiol. 5: 326.

Johns, A. T. 1951 b. The mechanism of propionic acid formation by propionibacteria. J. Gen. Microbiol. 5: 337.

Johns, A. T. 1951 c. Isolation of a bacterium, producing propionic acid, from the rumen of sheep. J. Gen. Microbiol. 5: 317.

Johns, A. T. 1952. The mechanism of propionic acid formation by *Clostridium propionicum*. J. Gen. Microbiol. 6: 123.

Johnson, R. R., B. A. Dehority, and O. G. Bentley. 1958. Studies on the *in vitro* rumen procedure: improved inoculum preparation and the effects of volatile fatty acids on cellulose digestion. J. Animal Sci. 19: 545.

Kaziro, Y., E. Leone, and S. Ochoa. 1960. Biotin and propionyl carboxylase. Proc. Natl. Acad. Sci. U.S. 46: 1319.

Kaziro, Y., L. F. Hass, P. D. Boyer, and S. Ochoa. 1962. Mechanism of the propionyl carboxylase reaction. II. Isotopic exchange and tracer experiments. J. Biol. Chem. 237: 1460.

Kitts, W. D., and L. A. Underkofler. 1954. Hydrolytic products of cellulose and the cellulolytic enzymes. J. Agr. Food Chem. 2: 639.

Krebs, H. A., and L. V. Eggleston. 1941. Biological synthesis of oxaloacetic acid from pyruvic acid and carbon dioxide. 2. The mechanism of carbon dioxide fixation in propionic acid bacteria. Biochem. J. 35: 676.

Ladd, J. N. 1959. The fermentation of lactic acid by a gram-negative coccus. Biochem. J. 71: 16.

Ladd, J. N., and D. J. Walker. 1959. The fermentation of lactate and acrylate by the rumen micro-organism LC. Biochem. J. 71: 364.

Leaver, F. W., and H. G. Wood. 1953. Evidence from fermentation of labeled substrates which is inconsistent with present concepts of the propionic acid fermentation. J. Cell. Comp. Physiol. 41: supple. 1: 225.

Lionetti, F. J., W. L. McLellan, and F. Communale. 1959. Inhibition of oxygen consumption in human erythrocytes by hexetidine. Biochem. Pharmacol. 2: 226.

Lionetti, F. J., N. C. Telles, and N. C. Avery. 1960. Inhibition of dehydrogenases of human leukocytes by hexetidine. Biochem. Pharmacol. 5: 219.

MacKay, V. G. 1963. Effects of inorganic salts on rumen volatile fatty acid production and percent fat in milk. Ph.D. thesis, Univ. of Alta.

Mazumder, R., T. Sasakawa, Y. Kaziro, and S. Ochoa. 1962. Metabolism of propionic acid in animal tissues. IX. Methylmalonyl coenzyme A racemase. J. Biol. Chem. 237: 3065.

Mazumder, R., T. Sasakawa, and S. Ochoa. 1963. Metabolism of propionic acid in animal tissues. X. Methylmalonyl coenzyme A mutase holoenzyme. J. Biol. Chem. 238: 50.

McCormick, N. G., E. J. Ordal, and H. R. Whiteley. 1962 a. Degradation of pyruvate by Micrococcus lactilyticus. I. General properties of the formate-exchange reaction. J. Bacteriol. 83: 887.

McCormick, N. G., E. J. Ordal, and H. R. Whiteley. 1962 b. Degradation of pyruvate by Micrococcus lactilyticus. II. Studies of cofactors in the formate-exchange reaction. J. Bacteriol. 83: 899.

Meites, S., R. C. Burrell, and T. S. Sutton. 1951. Factors influencing the in vitro digestion of cellulose by rumen liquor in the presence of an antiseptic. J. Animal Sci. 10: 203.

Nasr, H. 1950. Amylolytic activity in the rumen of the sheep. J. Agr. Sci. 40: 308.

Olson, R. E., O. N. Miller, Y. J. Topper, and F. J. Stare. 1948. The effects of vitamin deficiencies upon the metabolism of cardiac muscle in vitro. II. The effect of biotin deficiency in ducks with observations on the metabolism of radioactive carbon-labeled succinate. J. Biol. Chem. 175: 503.

Ochoa, S., A. Mehler, M. L. Blanchard, T. H. Jukes, C. E. Hoffman, and M. Regan. 1947. Biotin and carbon dioxide fixation in liver. J. Biol. Chem. 170: 413.

Ochoa, S., A. Mehler, and A. Kornberg. 1947. Reversible oxidative decarboxylation of malic acid. J. Biol. Chem. 167: 871.

Ochoa, S., A. H. Mehler, and A. Kornberg. 1948. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. I. Isolation and properties of an enzyme from pigeon liver catalyzing the reversible oxidative decarboxylation of l-malic acid. J. Biol. Chem. 174: 979.

Ochoa, S., J. B. V. Salles, and P. J. Ortiz. 1950. Biosynthesis of dicarboxylic acids by carbon dioxide fixation. III. Enzymatic synthesis of l-malic acid by reductive carboxylation of pyruvic acid. J. Biol. Chem. 187: 863.

Payes, B., and G. G. Laties. 1963. The inhibition of several tricarboxylic acid cycle enzymes by γ-hydroxy-α-ketoglutarate. Biochem. and Biophys. Res. Comm. 6: 460.

Pazur, J. H., T. Budovich, E. W. Shuey, and C. E. Georgi. 1957. The hydrolysis of xylan and xylooligosaccharides by ruminal enzymes. Arch. Biochem. and Biophys. 70: 419-425.

Pazur, J. H., E. W. Shuey, and C. E. Georgi. 1958. The conversion of D-xylose into volatile organic acids by rumen bacteria. Arch. Biochem. and Biophys. 77: 387.

Peel, J. L. 1960. The breakdown of pyruvate by cell-free extracts of the rumen micro-organism LC. Biochem. J. 74: 525.

Rojers, E. F. 1962. Thiamine antagonists. Ann. N. Y. Acad. Sci. 98: 412.

Scherr, G. H., and M. E. Rafelson, Jr. 1962. The directed isolation of mutants producing increased amounts of metabolites. J. Appl. Bacteriol. 25: 187.

Seaman, G. R., and M. D. Naschke. 1956. Reversible cleavage of succinate by extracts of tetrahymena. J. Biol. Chem. 217: 1.

Shuster, C. W., and F. Lynen. 1960. The role of cofactors and enzyme-bound biotin in the CO_2-pyruvate exchange reactions. Biochem. and Biophys. Res. Comm. 3: 350.

Sijpesteijn, A. K., and S. R. Elsden. 1952. The metabolism of succinic acid in the rumen of the sheep. Biochem. J. 52: 41.

Siu, P. M. L. 1962 a. An investigation of p-enolpyruvic carboxylase. Federation Proc. 21: 244.

Siu, P. M. L. 1962 b. Carbon dioxide fixation by phosphopyruvate carboxylase from spinach. Biochim. Biophys. Acta 63: 520.

Siu, P. M. L., and H. W. Wood. 1962. Phosphoenolypyruvic carboxytransphosphorylase, a CO_2 fixation enzyme from propionic acid bacteria. J. Biol. Chem. 237: 3044.

Siu, P. M. L., H. G. Wood, and R. L. Stjernholm. 1961. Fixation of CO_2 by phosphoenolypyruvic carboxytransphosphorylase. J. Biol. Chem. 236: PC21.

Stadtman, E. R., and P. R. Vagelos. 1958. Propionic acid metabolism. Intern. Union Biochem. Symp. Ser. 2: 86.

Steel, R. G. D., and J. H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Book Co. Inc., New York. 481 p.

Stern, J. R. 1963. Enzymic reactions of pyruvate in Clostridium kluyveri. Federation Proc. 22: 355.

Stjernholm, R., and H. G. Wood. 1961. Methylmalonyl isomerase. II. Purification and properties of the enzyme from propionibacteria. Proc. Natl. Acad. Sci. U.S. 47: 303.

Stjernholm, R., H. G. Wood, S. H. G. Allen, and B. Jacobson. 1962. Purification of transcarboxylase containing radioactive biotin. Federation Proc. 21: 244.

Swick, R. W., and H. G. Wood. 1960. The role of transcarboxylation in propionic acid fermentation. Proc. Natl. Acad. Sci. U.S. 46: 28.

Topper, Y. J., and D. Stetten, Jr. 1954. Formation of "acetyl" from succinate by rabbit liver slices. J. Biol. Chem. 209: 63.

Utter, M. F., and D. B. Keech. 1960. Formation of oxaloacetate from pyruvate and CO_2. J. Biol. Chem. 235: PC17.

Utter, M. F., and K. Kurahashi. 1953. Mechanism of action of oxalacetic carboxylase from liver. J. Am. Chem. Soc. 75: 758.

Utter, M. F., and K. Kurahashi. 1954. Purification of oxaloacetic carboxylase from chicken liver. J. Biol. Chem. 207: 787.

Van Niel, C. B. 1953. Introductory remarks on the comparative biochemistry of microorganisms. J. Cell. Comp. Physiol. 41: supple. 1: 11.

Whiteley, H. R. 1953 a. Cofactor requirements for the decarboxylation of succinate. J. Amer. Chem. Soc. 75: 1518.

Whiteley, H. R. 1953 b. The mechanism of propionic acid formation by succinate decarboxylation. I. The activator of succinate. Proc. Natl. Acad. Sci. U.S. 39: 772.

Whiteley, H. R. 1953 c. The mechanism of propionic acid formation by succinate decarboxylation. II. The formation and decarboxylation of succinyl-CoA. Proc. Natl. Acad. Sci. U.S. 39: 779.

Whiteley, H. R., and N. G. McCormick. 1963. Degradation of pyruvate by Micrococcus lactilyticus. III. Properties and cofactor requirements of the carbon dioxide-exchange reaction. J. Bacteriol. 85: 382.

Wood, H. G., S. H. G. Allen, R. Stjernholm, and B. Jacobson. 1963. Transcarboxylase. III. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase containing tritiated biotin. J. Biol. Chem. 238: 547.

Wood, H. G., and F. W. Leaver. 1953. CO_2 turnover in the fermentation of 3,4,5 and 6 carbon compounds by the propionic acid bacteria. Biochim. Biophys. Acta. 12: 207.

Wood, H. G., and R. Stjernholm. 1961. Transcarboxylase. II. Purification and properties of methylmalonyl-oxaloacetic transcarboxylase. Proc. Natl. Acad. Sci. U.S. 47: 289.

Wood, H. G., R. Stjernholm, and F. W. Leaver. 1956. The role of succinate as a precursor of propionate in the propionic acid fermentation. J. Bacteriol. 72: 142.

Wood, H. G., C. H. Werkman, A. Hemingway, and A. O. Nier. 1941. Heavy carbon as a tracer in heterotrophic carbon dioxide assimilation. J. Biol. Chem. 139: 365.

Woolley, D. W. 1952. A study of antimetabolites. John Wiley and Sons, Inc., New York. 269 p.

TABLE 1

Effects of glucose on cellulose digestion and VFA production in the presence and absence of added biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 18	complete + glucose	145.2	1.87	1.51	0.37
	complete	141.1	1.65	1.29	0.18
	difference	4.1	0.22	0.22	0.19
	biotin deleted + glucose	38.8	1.27	0.68	0.48
	biotin deleted	47.8	1.15	0.55	0.16
	difference	-9.0	0.12	0.13	0.32
Run 19	complete + glucose	165.2	1.47	1.89	0.31
	complete	167.3	1.49	1.56	0.19
	difference	-2.1	-0.02	0.33	0.12
	biotin deleted + glucose	48.9	1.13	0.96	0.33
	biotin deleted	44.4	0.90	0.54	0.21
	difference	4.5	0.23	0.42	0.12
Run 21	complete + glucose	139.1	1.58	1.73	0.32
	complete	148.2	1.49	1.48	0.18
	difference	-9.1	0.09	0.25	0.14
	biotin deleted + glucose	27.6	0.93	0.72	0.31
	biotin deleted	27.2	0.84	0.48	0.16
	difference	0.2	0.09	0.24	0.15

TABLE 2

Effects of 3-phosphoglycerate on cellulose digestion and VFA production in the presence and absence of added biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 20	complete + 3-PG[1]	112.2	1.58	1.11	0.22
	complete	151.5	1.43	1.59	0.21
	difference	-39.3	0.15	-0.48	0.01
	biotin deleted + 3-PG	21.8	1.02	0.43	0.29
	biotin deleted	11.9	0.55	0.25	0.17
	difference	9.9	0.47	0.18	0.12
Run 24	complete + 3=PG	127.9	2.36	1.23	0.19
	complete	145.2	1.85	1.53	0.19
	difference	-17.3	0.51	-0.30	0
	biotin deleted + 3-PG	36.9	1.83	0.51	0.22
	biotin deleted	31.9	1.06	0.43	0.14
	difference	5.0	0.77	0.08	0.08

[1] 3-phosphoglycerate

TABLE 3

Effects of pyruvic acid on cellulose digestion and VFA production in the presence of added biotin and in biotin deficiency

	Treatment	Cellulose digested mg	VFA concentrations (mmole/100 ml) Acetate	Propionate	Butyrate
Run 15	complete + pyruvic acid	178.7	2.43	1.91	0.17
	complete	180.9	1.69	1.80	0.13
	difference	-2.2	0.74	0.11	0.04
	12 units avidin + pyruvic acid	74.7	1.72	0.84	0.13
	12 units avidin	45.0	0.91	0.65	0.08
	difference	29.7	0.81	0.19	0.05
Run 18	complete + pyruvic acid	151.7	2.37	1.41	0.23
	complete	141.1	1.65	1.29	0.18
	difference	10.6	0.72	0.12	0.05
	biotin deleted + pyruvic acid	66.5	1.82	0.68	0.23
	biotin deleted	47.8	1.15	0.55	0.16
	difference	18.7	0.67	0.13	0.07
Run 19	complete + pyruvic acid	174.0	2.01	1.50	0.26
	complete	167.3	1.49	1.56	0.19
	difference	6.7	0.52	-0.06	0.07
	biotin deleted + pyruvic acid	57.8	1.61	0.67	0.31
	biotin deleted	44.4	0.90	0.54	0.21
	difference	13.4	0.71	0.13	0.10

TABLE 4

Effects of lactic acid on cellulose digestion and VFA production in the presence and absence of added biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 20	complete + lactic acid	162.4	1.40	1.78	0.40
	complete	151.5	1.43	1.59	0.21
	difference	10.9	-0.03	0.19	0.19
	biotin deleted + lactic acid	20.3	0.69	0.35	0.35
	biotin deleted	11.9	0.55	0.25	0.17
	difference	8.4	0.14	0.10	0.18
Run 21	complete + lactic acid	139.9	1.51	1.57	0.33
	complete	148.2	1.49	1.48	0.18
	difference	-8.3	0.02	0.09	0.15
	biotin deleted + lactic acid	33.8	0.93	0.65	0.33
	biotin deleted	27.2	0.84	0.48	0.16
	difference	6.6	0.09	0.17	0.17
Run 26	complete + lactic acid	153.6	2.31	1.73	0.41
	complete	144.6	2.00	1.46	0.15
		9.0	0.31	0.27	0.26
	biotin deleted + lactic acid	87.5	1.99	1.25	0.43
	biotin deleted	64.9	1.59	0.75	0.14
	difference	22.6	0.40	0.50	0.29

TABLE 5

Effects of oxaloacetic acid on cellulose digestion and VFA production in the presence and absence of added biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 23	complete + OAA[1]	91.6	2.35	0.94	0.19
	complete	146.0	1.84	1.39	0.19
	difference	-54.4	0.51	-0.45	0
	biotin deleted + OAA	49.0	2.01	0.63	0.20
	biotin deleted	60.5	1.33	0.67	0.18
	difference	-11.5	0.68	-0.04	0.02
Run 24	complete + OAA	96.6	2.13	1.01	0.17
	complete	145.2	1.88	1.84	0.14
	difference	-48.6	0.25	-0.83	0.03
	biotin deleted + OAA	33.5	1.89	0.54	0.21
	biotin deleted	31.9	1.06	0.43	0.14
	difference	1.6	0.83	0.09	0.07

[1]oxaloacetic acid

TABLE 6

Effect of malic acid on cellulose digestion and VFA production in the presence and absence of added biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 20	complete + malic acid	144.9	1.51	1.91	0.25
	complete	151.5	1.43	1.59	0.21
	difference	-6.6	0.08	0.32	0.04
	biotin deleted + malic acid	22.4	0.98	0.84	0.30
	biotin deleted	11.9	0.55	0.25	0.17
	difference	10.5	0.43	0.59	0.13
Run 21	complete + malic acid	119.8	1.44	1.62	0.20
	complete	148.2	1.49	1.48	0.18
	difference	-28.4	-0.05	0.14	0.02
	biotin deleted + malic acid	30.5	1.16	0.93	0.23
	biotin deleted	27.2	0.84	0.48	0.16
		3.3	0.32	0.45	0.07
Run 23	complete + malic acid	144.3	2.27	2.03	0.23
	complete	146.0	1.84	1.39	0.19
	difference	-1.7	0.43	0.64	0.04
	biotin deleted + malic acid	84.2	1.63	1.43	0.17
	biotin deleted	60.5	1.33	0.67	0.18
	difference	23.7	0.30	0.76	-0.01
Run 26	complete + malic acid	160.2	2.46	2.24	0.18
	complete	144.6	2.00	1.46	0.18
	difference	15.6	0.46	0.78	0
	biotin deleted + malic acid	76.3	1.86	1.56	0.15
	biotin deleted	64.9	1.59	0.75	0.14
	difference	11.4	0.27	0.81	0.01

TABLE 7

Effects of fumaric acid on cellulose digestion and VFA production in the presence and absence of biotin

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetate	Propionate	Butyrate
Run 23	complete + fumaric acid	126.0	2.11	1.72	0.15
	complete	146.0	1.84	1.39	0.19
	difference	-20.0	0.27	0.33	-0.04
	biotin deleted + fumaric acid	44.9	1.62	0.80	0.13
	biotin deleted	60.5	1.33	0.67	0.18
	difference	-15.6	0.29	0.13	-0.05
Run 24	complete + fumaric acid	140.7	1.88	1.84	0.14
	complete	145.2	1.85	1.53	0.19
	difference	-4.5	0.03	0.31	-0.05
	biotin deleted + fumaric acid	41.1	1.67	1.11	0.18
	biotin deleted	31.9	1.06	0.43	0.14
	difference	9.2	0.61	0.68	0.04

TABLE 8

Effects of succinic acid on cellulose digestion and VFA production in the presence of added biotin and in biotin deficiency

	Treatment	Cellulose digested mg	VFA concentrations (mmoles/100 ml) Acetic	Propionic	Butyrate
Run 15	complete + succinic acid	178.0	1.59	2.68	0.13
	complete	180.9	1.69	1.80	0.13
	difference	-2.9	-0.10	0.88	0
	12 units avidin + succinic acid	57.4	0.98	1.71	0.10
	12 units	45.0	0.91	0.65	0.08
	difference	12.4	0.07	1.06	0.02
Run 18	complete + succinic acid	149.1	1.61	2.19	0.17
	complete	141.1	1.65	1.29	0.18
	difference	8.0	-0.04	0.90	-0.01
	biotin deleted + succinic acid	49.0	1.22	1.40	0.20
	biotin deleted	47.8	1.15	0.55	0.16
	difference	1.2	0.07	0.85	0.04

APPENDIX B

Calculation of VFA Precursor Conversions

The difference in the VFA content of a control fermentation tube and one to which had been added a VFA precursor could not be considered a true indication of the conversion of that precursor to the individual fatty acids, because there was always a difference between the amount of cellulose digested in the control fermentation tubes and that digested in the treatment fermentation tubes. It was deemed necessary to estimated the quantity of each of the fatty acids produced from the cellulose digested in each treatment (basal VFA production). This calculation was carried out in the following manner.

It was previously observed (Experiment II) that the concentration of the fatty acids was a linear function of cellulose digested. In each fermentation in which the conversion of metabolic intermediates to VFA was to be studied there were 3 complete medium control tubes and 3 biotin-deleted control tubes. There was invariably a wide range in the cellulose digestion between these two controls. Therefore, regression lines for the production of acetate, propionate and butyrate upon cellulose digested could be calculated from the two sets of controls, for each run. Using these regression lines, the basal VFA concentrations of the treatment tubes were readily calculated.

It was thought that a comparison of the actual VFA production in a treatment tube to its basal VFA production would provide a realistic picture of the conversion of the intermediate to VFA, but this was not the case. It was found that the difference between the total VFA recovery in a treatment tube compared to its basal production would vary greatly within and between runs. It was evident that one could not compare the conversion

of a precursor in the presence and absence of biotin within a run, if, in one case the recovery of VFA from the precursor was 1.00 mmole per 100 ml, while in the other case the VFA recovery from the precursor fermentation was only 0.50 mmoles per 100 ml. It was observed that the recovery of precursor as VFA could not be related to the amount of cellulose digested, and did not appear to be at all related to the variation and inconsistencies in the results obtained. It was therefore decided that, in order to be able to make comparisons of conversions in the presence and absence of biotin, and between precursors, the individual fatty acids produced from the precursor would have to be expressed as a fraction of the total VFA produced from that precursor. This calculation was accomplished in the following manner:

The average acetate, propionate and butyrate concentrations of a precursor treatment were designated as Ac(t), Pr(t) and Bu(t) respectively, while the corresponding calculated basal concentrations were identified as Ac(b), Pr(b) and Bu(b). The total VFA units produced from the intermediate was calculated using the following formula: $\{Ac(t) - Ac(b)\} + \{Pr(t) - Pr(b)\} + 2\{Bu(t) - Bu(b)\}$. Butyrate was weighted because it is assumed that one butyrate unit would be equivalent to two acetate units. The proportion of acetate or of propionate in the total VFA units was calculated using the general formula; proportion of $X = \frac{X(t) - X(b)}{\text{total VFA units}}$, where X refers to the acid under consideration. The proportion of butyrate was calculated using the formula; proportion of butyrate $= 2\frac{\{Bu(t) - Bu(b)\}}{\text{total VFA units}}$. These proportional recovery values were used in the description of Experiment III in the body of this thesis. The values were calculated from the data of Appendix A, Tables 1 to 8.